Evaluation of telomerase activity and telomerase inhibitors in Head and Neck cancer

ADESOLA A ADEKUNLE

Submitted for the Degree of Doctor of Philosophy

Faculty of Life Sciences

University of Bradford

2019

Abstract

Cancer is a major cause of morbidity and mortality with increasing incidence worldwide. Early detection of cancers and better treatments would improve the outcome for patients. The overall 5-year survival rates of head and neck squamous cell carcinoma have not improved in the past several decades due to diagnosis at advanced stages and recurrent disease. Early detection and improved chemotherapy drugs are two key areas that are required to help to improve the prognosis for this disease.

This thesis focuses on the enzyme telomerase which is known to contribute to one of the hallmarks of cancer (immortality). Elevated telomerase activity has been observed in the majority of cancer cells but not in most normal human cells so there is an opportunity to use telomerase as a biomarker for disease. This first part of this study assessed telomerase activity in saliva and tissues of head and neck squamous cell cancer patients. The Telomerase PCR-ELISA kit was used to assess telomerase activity in the saliva of patients with confirmed oral carcinomas and its expression was analysed in paraffin embedded tissue using immunohistochemistry (IHC). Whilst telomerase was detected in cell lines, no telomerase activity was detected in saliva samples from patients but was detectable in IHC specimens.

The second part of the study focused on the pharmacological evaluation of a series of small molecule G–quadruplex DNA binding agents as potential telomerase inhibitors. A total of 19 telomerase inhibitors were identified but of these, only 4 were specific inhibitors of telomerase. These compounds also caused toxicity to cell lines following a 2 hour drug exposure at doses that also inhibit telomerase activity. Further studies are required to explore these compounds further.

In conclusion, the results of this study have demonstrated that detection of telomerase activity I the saliva of patients with oral cancers is unlikely to be useful in terms of detecting oral cancers before symptoms of the disease are clinically manifest. A series of novel and specific inhibitors of telomerase have been identified and further studies are required to develop these compounds further.

Table of contents

Acknowledgements

I would like to thank my supervisors, Professor Roger Phillips for his help, endless encouragement and support throughout this research project and Dr Sue Parkin for her support and guidance. Thanks also to Mr David Watt at the Bradford Royal Infirmary for clinical supervision of the first part of the project.

I also wish to thank Professor Anne Graham for her support in the completion of the project.

Many thanks are due Dr Richard Wheelhouse for providing the compounds used in the study, advice and guidance.

My gratitude extends to Dr Angelika Burger formerly at the Wayne State University School Medicine who I met with and provided invaluable advice towards the further direction of my study. Dr Burger was a gifted researcher who passed away in May 2011 but has left a body of work that has contributed to research advancement in cancer.

I am grateful to the Burns and Plastic Surgery Research Unit and Professor David Sharpe for giving me the opportunity to undertake this research.

Finally, I would like to thank my family whose support has been invaluable. To my parents, brothers and sister who continued to support and encourage me. To my gentle and loving husband Daniel, my children Jonathan, Rachael, Abigail and Anna who have endured so much over all these years and without whom I would not be where I am.

Abbreviations

Anti- DIG-POD	antibody against digoxigenin
BSA	bovine serum albumin
DIG	digoxigenin
DNA	deoxy ribo nucleic acid
DMEM	Dulbecco's Modified Eagles's Medium
DMSO	dimethyl sulphoxide
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunoadsorbent assay
hTERT	human telomerase reverse transcriptase
hTR	human telomerase RNA
HBSS	Hanks balanced salt solution
HNSCC	head and neck squamous cell cancer
HUVEC	human umbilical vein endothelial cells
PCR	polymerase chain reaction
PBS	phosphate buffered saline
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
TBST	tris-buffered saline + Tween 20
TMB	tetra methyl benzidine
TRAP	telomeric repeat amplification protocol
VEGF	vascular endothelial growth factor

Posters / Presentations given derived from this thesis

Novel small inhibitors of Telomerase

Adekunle A, Evans HR, Phillips VA, Pletsas D, Wheelhouse RT, Parkin SM, Watt DAL, Sharpe DTS, Phillips RM

20th EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics October 21-24, 2008. Geneva, Switzerland.

Telomerase activity in head and neck squamous cell carcinoma and its inhibition by novel compounds

Adekunle A, Wheelhouse RT, Parkin SM, Watt DAL, Sharpe DTS, Phillips RM

British Association of Plastic Reconstructive and Aesthetic Surgeons winter meeting December 3-5, 2008.London, UK

This thesis is dedicated to the 56 people who lost their lives and the over 200 injured in the Bradford City Football Club fire disaster in May 1985

1 INTRODUCTION

1.1 Cancer

The term cancer usually refers to malignant tumours that have the ability to invade and destroy surrounding normal tissues and spread to distant sites. A tumour is a term used to describe a diverse group of conditions associated with uncontrolled cell replication. Tumours that do not metastasize and remain confined to a specific site are classified as benign tumours. Cancers are formed when cells escape the normal growth regulating processes to extend beyond the normal confines of that tissue

The tissue of origin gives the cancer distinguishing characteristics therefore pathologically, cancers are classified as: (i) Carcinoma which originate from epithelial cells in the skin, or tissues that line or cover internal organs, (ii) Sarcoma – originating in bone, cartilage, fat, muscle, blood vessels, or other connective tissue (iii) Leukemia – originating in blood forming tissues such as bone marrow, which causes large numbers of abnormal blood cells to be produced into the blood stream and (iv) Lymphoma - originate in the cells of the immune system.

In the UK, more than 200 different types of cancers occur but four of them – breast, lung, colorectal and prostate account for over half of the new cases (figure 1). The 20 most commonly diagnosed cancers in the UK are shown in figure 2 (UK cancer incidence statistics 2015, Cancer Research UK)

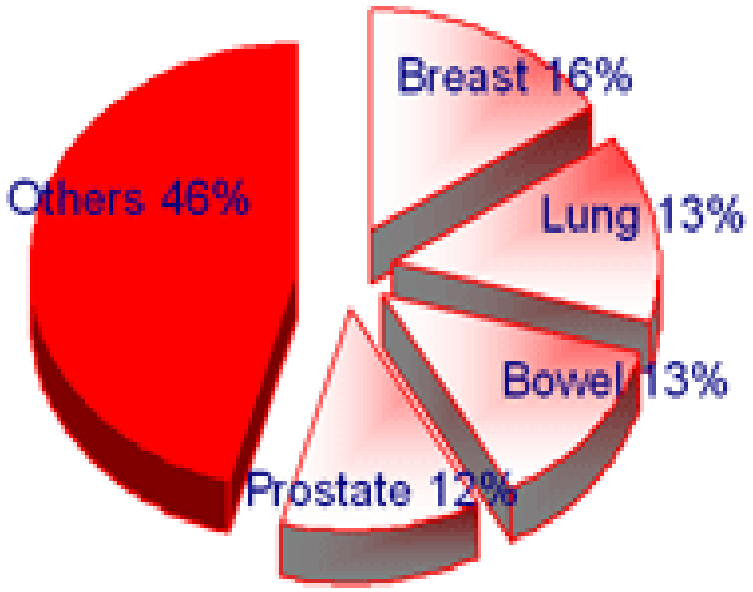


Figure 1: Cancer incidence in the UK in 2015 (Cancer research UK)

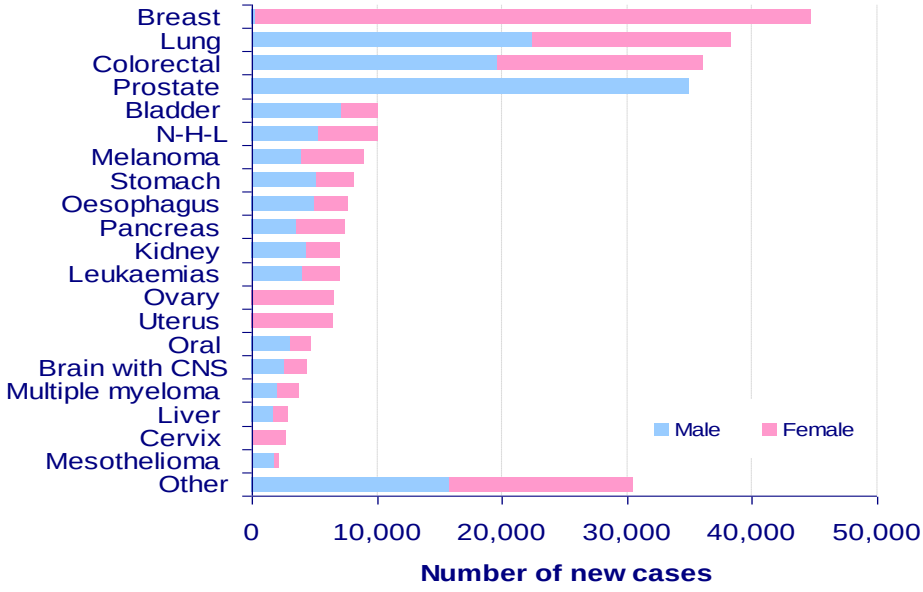


Figure 2: The 20 most commonly diagnosed cancers (excluding non melanoma skin cancer) in the UK 2015 (Cancer research UK).

Cancer is a major disease burden worldwide and approximately 14 million people were diagnosed with cancer in 2012. Worldwide, it is a leading cause of death, accounting for 8.8 million deaths in 2015 and deaths from cancer are expected to rise by about 70% over the next 2 decades (WHO Cancer fact sheet 2018). Cancer is responsible for more than 1 in 4 of all deaths in the UK, and more than 1 in 3 people in the UK will develop some form of cancer in their lifetime (Cancer statistics key facts, Cancer research UK 2015). The economic impact of cancer is significant and total annual economic cost in 2010 was estimated at approximately US $1.16 trillion (Stewart and Wild 2014). It is therefore a condition with major socioeconomic impact requiring great efforts to improve current strategies for detection and treatment.

1.2 Aetiology

The precise cause of cancers is unknown, but most cancers are thought to have a multifactorial aetiology. Cancers are thought to occur due to alterations to the DNA sequence (mutation), caused by either genetic or environmental factors. Genetic factors such as familial disposition and chromosomal abnormalities are thought to be associated with increased incidence of cancer. External agents which have been identified in the cause of cancer include environmental factors such as irradiation, chemicals (such as asbestos and tobacco) and infectious agents (viruses).

Nowell (1976) proposed that most cancers arise from a single cell, and progression results from the accumulation of genetic changes within the original clone which confer a growth advantage in the cancer cells over normal cells. In the past 30 years, there has been a tremendous increase in information on the molecular basis of cancer development, made possible by the breakthroughs in methodologies of

molecular biology in the early 1980's and 90's. It has been shown that cancers typically arise from alterations in pathways that regulate cellular signaling involving DNA damage response, cell cycle arrest and apoptosis. Normal growth control is regulated by two different types of genes: proto-oncogenes whose normal cellular function is to accelerate cell proliferation, and tumour suppressor genes, which function to inhibit cell proliferation. If this balance between proto-oncogenes and tumour suppressor genes is disturbed due to mutation in these genes, this could result in the development of cancer. The mutations have been shown to occur in a multistep process over a long period of time. The most intensely studied model of tumor progression is colorectal cancer, a model proposed by Bert Vogelstein (Fearon and Vogelstein 1990), which links histological features in the progression of colorectal cancer to specific genetic lesions. Mutation of the familial polyposis gene APC constitutes the initiating event, additional genetic lesions are associated with polyps of increasing severity, and finally, inactivation of p53 commonly marks the transition to invasive colorectal cancer. In other tumor types, this progression may be driven by the loss of different tumor suppressor genes or by a different cumulative order in the inactivation of the same genes, including p53. The model of tumor progression of head and neck cancer will be discussed in subsequent chapters. Hanahan and Weinberg (2000) proposed that during the development of cancer, cells acquire a number of characteristics or traits that they called 'hallmarks' - these are summarized in figure 3. In their original paper in 2000 (figure 3), six hallmarks were defined. These include: (i) self sufficiency in growth signaling (ii) insensitivity to antigrowth signals (iii) evading apoptosis (iv) limitless replicative potential (v) sustained angiogenesis and (vi) tissue invasion and metastasis. In an update to their 2000 paper, Hanahan and Weinberg (2011) described two emerging hallmarks of

cancer and two enabling characteristics (figure 4). The emerging hallmarks were: metabolic reprogramming and evasion of immunsurveilance and the enabling characteristics were: genetic instability and inflammation (Hanahan and Weinberg 2011).

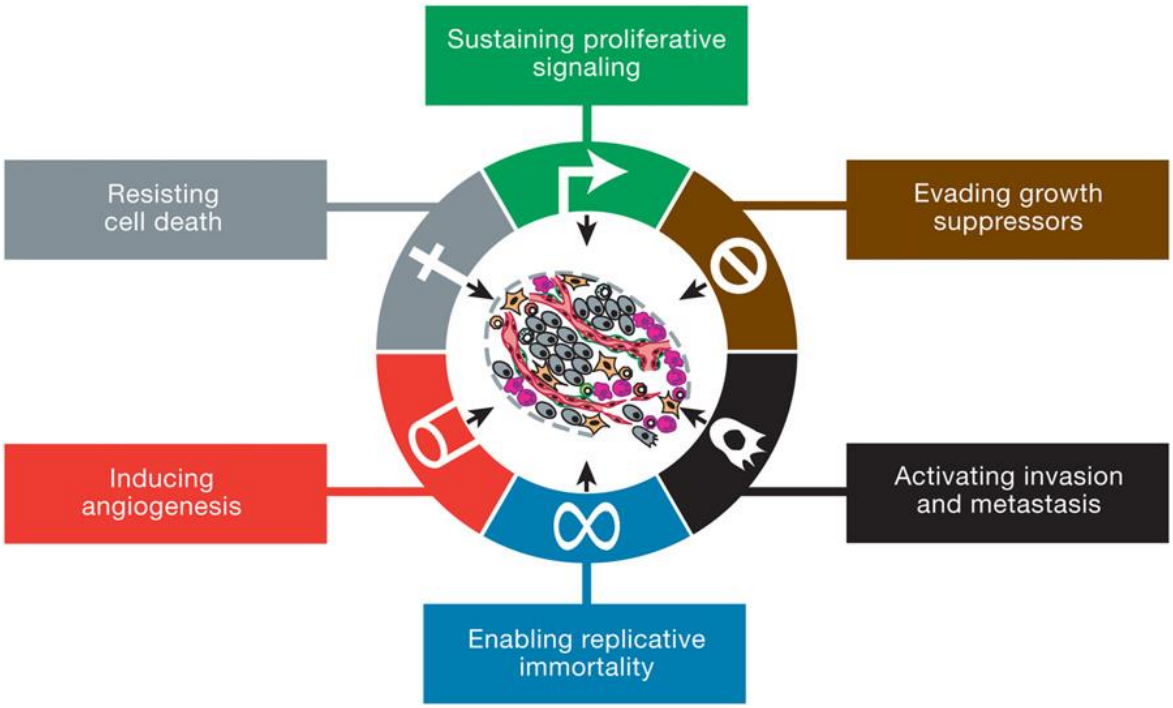


Figure 3 The hallmarks of cancer as originally described by Hanahan and Weinberg in 2000

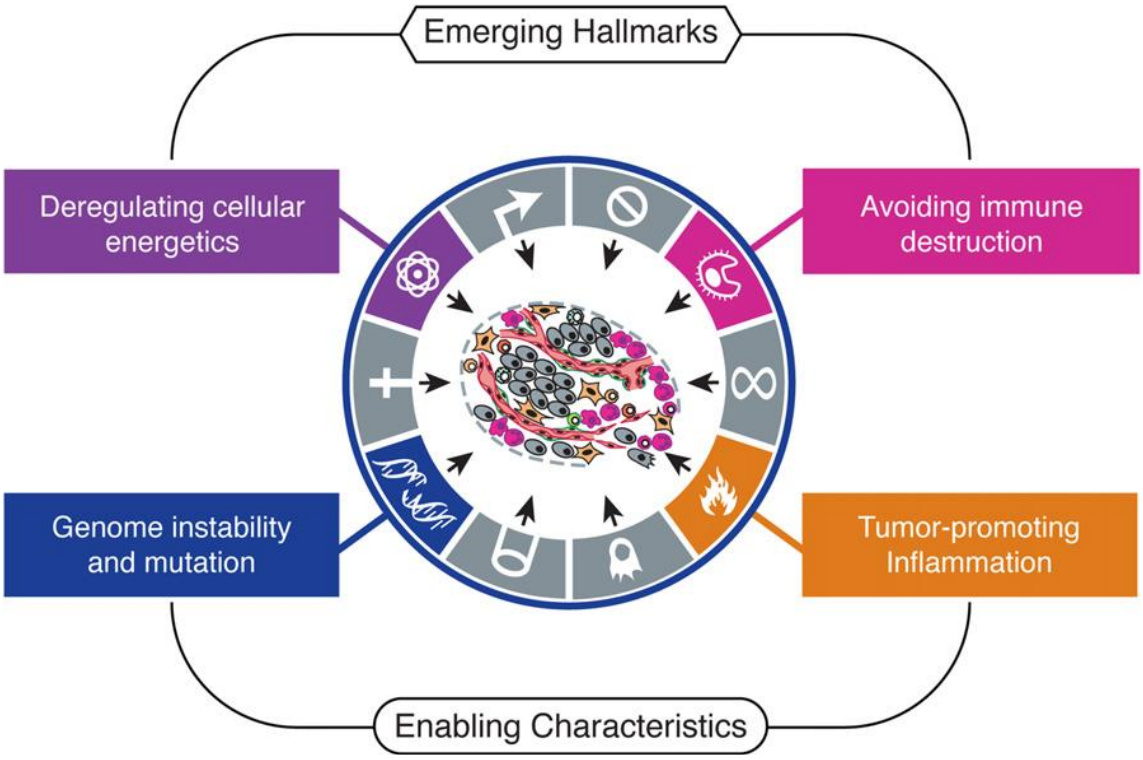


Figure 4. Emerging hallmarks and enabling characteristics (Hanahan and Weinberg 2011)

Among these hallmarks, the acquisition of unlimited replicative potential is a key step to ensure expansive tumour growth (Olaussen et al. 2006). An enzyme called telomerase has been identified as providing cancer cells with unlimited replicative capacity described. Hayflick (1997) demonstrated that cultured cells have a finite replicative potential and undergo a certain number of doublings, after which they stop growing (a process termed senescence) or undergo cell death. On the other hand, cancer cells in culture have the ability to multiply without limit and this characteristic represents the hallmark of replicative immortality of cancer cells. Telomerase is a ribonucleoprotein complex with enzymatic activity responsible for the maintenance of telomeres, which cap and protect the ends of chromosomes. The thesis described here will be focusing on telomerase, its detection and also its position as a target for cancer therapeutics. The structure and function of telomerase

will be discussed in subsequent chapters but initially, context is provided by briefly reviewing key aspects of cancer diagnosis, treatment and drug development.

1.3 Diagnosis of cancer

The diagnosis of cancer is made from clinical history and investigation of symptoms. Clinical manifestation of cancer varies depending on the local effect of the primary tumour and effects on any site of metastatic spread. Investigations include routine blood tests, tests for tumour markers in body fluids, genetic tests and radiological investigations. Tissue diagnosis is made from tissue samples obtained from abnormal areas which are subjected to histological analysis. Histological features of cancerous growths include cellular de-differentiation, invasion of adjacent tissues, and microscopic evidence of metastasis. A cancerous tissue shows cellular abnormalities such as: local increase in cell number, loss of the normal regular arrangement of cells, variation in cell shape and size, increase in nuclear size and density of staining, increase in mitotic activity, and presence of abnormal mitosis and chromosomes. Variability however can occur amongst histopathologists in the interpretation of histological findings which can limit in diagnostic histopathology (Soyer et al. 2005).

1.4 Cancer therapy

The ideal way to defeat cancer would be to prevent it and education of individuals about environmental factors and avoidance of them would play a role reducing the incidence. Coupled with this, early detection of cancer before it has metastasized would also contribute significantly to improving survival as surgery and radiotherapy

are effective at treating localised tumours. The reality is however that many cancers have spread by the time they are diagnosed and others (leukaemia / lymphoma) are disseminated from the onset. Systemic based therapies are therefore required, and the following subsections provide an overview of current treatment strategies together with their limitations.

1.4.1 Surgery

Surgery offers a means of local treatment. It may have several components (i) tissue biopsy to establish diagnosis and (ii) removal of malignant disease with clear margins of normal tissue. The type of surgery required is determined by the type of tumour and anatomical site. This may include (i) wide local excision of the tumour mass, (ii) removal of part of an organ and surrounding tissue and (iii) removal of an entire organ. In the context of head and neck cancer, this may include for example, partial removal of the tongue in carcinoma of the tongue or laryngectomy (removal of the larynx) in carcinoma of the larynx. This may have resulting long term morbidity with impaired physiological functions of speech and swallowing. Organ preservation is therefore a goal of treatment.

1.4.2 Radiotherapy

This is the use of ionizing radiation to treat disease. Ionizing radiation may be delivered by X-ray beams, beams of ionizing particles (for example electrons) or by beta or gamma irradiation produced by the decay of radioactive isotopes. It offers a means of loco-regional control of cancer, covering a wider area and is less constrained by anatomical boundaries and surgical technique. It can be used alone or in combination with surgery and/or chemotherapy. In early stage head and neck cancer, it provides a highly effective means of cure.

1.4.3 Chemotherapy

Cancer chemotherapy is the use of cytotoxic agents to destroy cancer cells. Compared to surgery and radiotherapy, chemotherapy is a relatively new method of treatment having been developed in the 1940s following the successful use of mustard derived compounds in humans (Goodman and Gilman 1946). Surgery had been used to treat localized disease for more than a century and radiotherapy for a quarter of a century before the first chemotherapy was given to a patient. Chemotherapy is a systemic form of treatment using drugs that are often highly toxic since they are rarely selective for cancer cells. The common basis of the activity of these agents is that they kill dividing cells by either inhibition of DNA replication or by acting as mitotic poisons. Chemotherapy agents can be classified into (i) drugs acting on the structure of DNA such as antimetabolites, alkylating agents, intercalating agents and topoisomerase inhibitors, (ii) drugs acting on mitosis such as spindle poisons, (iii) signal transduction inhibitors, (iv) drugs inducing apoptosis and (v) drugs targeting the tumour vasculature (Neal and Hoskin 2009). Chemotherapy may be used alone but is more often used in combination with surgery or radiotherapy as an adjuvant to loco- regional treatment to enhance local control and attack potential sites of metastasis. One of the limitations in using chemotherapeutic agents is toxicity to normal tissues, as the majority of agents are non-specific in their action resulting in damage to both malignant and normal cells. Common limitation toxicities include bone marrow suppression, bowel toxicity, renal and neurological damage. Another drawback of chemotherapy is the development of drug resistance which can be an intrinsic property of the malignant cell or acquired by the cells after exposure to individual drugs. Development of resistance is the main cause of treatment failure following an initial good response to chemotherapy. The

concept of drug resistance and the recognition of the frequency with which it occurs underlie the use of drug combinations for systemic therapy. In head and neck cancers, multi-agent chemotherapy (along with radiotherapy), have been shown to improve survival rates (Garden et al 2001).

1.5 Target oriented approach to cancer therapy

Cancers arise from normal human cells, and chemotherapy is often highly toxic and rarely selective for cancer cells which have made the chemotherapeutic approach to the treatment of cancers challenging. In recent decades there have been advances in the knowledge of the molecular and biological traits of cancer cells which distinguish them from normal cells. These molecular and biochemical changes have therefore become potential targets for drug development. Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules that are involved in the growth, progression and spread of cancer. Rather than using broad based cancer treatments, focusing on specific molecular changes which are unique to a particular cancer, targeted cancer therapies may be more therapeutically beneficial for many cancer types (Baudino T. 2015). Current cancer research activities are focused on targeted drug development, with drugs designed to block tumour specific receptors and proteins and this has led to promising novel agents being evaluated. These new classes of cancer targeted drugs which have emerged can be categorized according to their respective effects on one or more of the hallmark capabilities. Figure 5 below is an illustration of the acquired capabilities of cancer cells as described by Hanahan and Weinberg (2011), showing the mechanism of action of drugs that interfere with the molecular processes (‘targets’), which enable cancer cells to acquire the hallmarks of cancer.

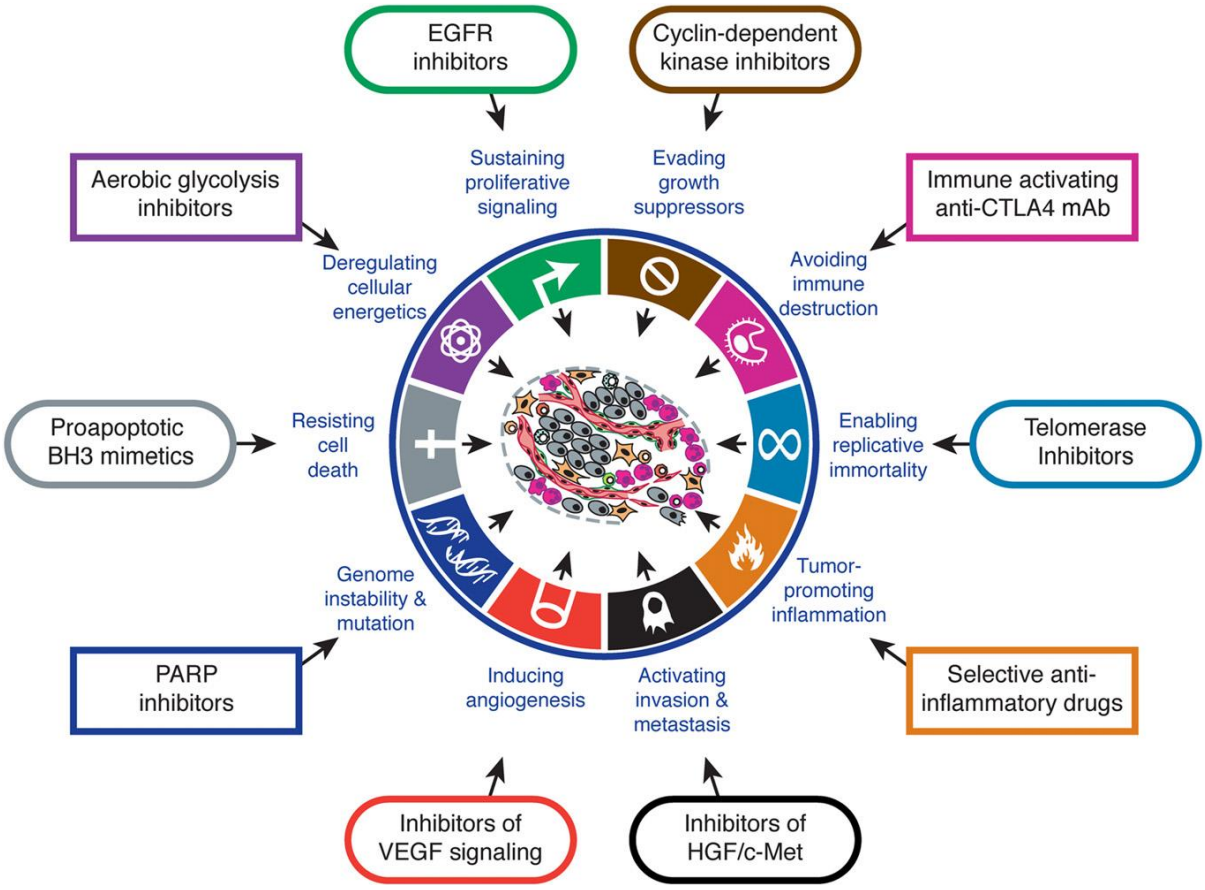


Figure 5 Therapeutic targeting of the hall marks of cancer (Hanahan and Weinberg, 2011)

. Most targeted therapies are either small molecule inhibitors or monoclonal antibodies. The most successful small molecule inhibitor so far has been Imatinib mesylate (Gleevec), which is a tyrosine kinase inhibitor that targets Bcr-Abl and one of the first targeted drugs to be developed. It is used in the treatment of chronic myeloid leukaemia and has revolutionised the treatment of these group of patients with improved 10 year survival rates (Hochhaus et al. 2017). It is also used in the treatment of gastrointestinal stromal tumours via its ability to inhibit c-kit. It has relatively few side effects and is a success story in the development of targeted therapies. An example of monoclonal antibodies includes inhibitors of angiogenesis (VEGF inhibitors) which in randomised control clinical trials (Murukesh et al 2010) have demonstrated an improvement in response, progression free survival or overall

survival when used to supplement conventional therapy, in colorectal cancer patients and breast cancer patients. Bevacizumab (Avastin) is an example of a VEGF inhibitor (Ranieri et al. 2006). Another class of targeted agents includes inhibitors of proliferative signalling - EGFR inhibitors (Dziadziuszko & Jassem. 2012) such as Erlotinib (Rosell et al. 2012), Gefitinib (Mitsudomi et al. 2010) and Cetuximab (Van Cutsem et al. 2015) which have also demonstrated improvement in response and progression free survival. However, an issue with most targeted drugs is that clinical responses have been transitory followed by relapses. Experimental evidence suggests that the targeted agent may not completely shut off a hallmark capability, allowing some cancer cells to survive with residual function, until they are able to develop adaptive resistance or reduce their dependence on a particular hallmark capability and become more dependent on another (Hanahan and Weinberg 2011; Azam et al 2010). The most common side effects seen with targeted therapies are diarrhoea and liver problems. Other side effects include skin problems, hypertension, clotting and wound healing problems.

Table 2 below shows examples of drugs that have been developed that interfere with the acquired capabilities necessary for tumour growth and development. The hallmark ‘enabling replicative mortality’ looking at telomerase and telomerase inhibitors will be the area of focus of this thesis. This will be discussed further in subsequent chapters.

Acquired capability	**Molecular mechanisms**	**Drugs**	**References**
Sustaining proliferative signaling	EGFR inhibitors (Dziadziuszko & Jassem 2012)	Gefitinib Erlotinib Cetuximab	Mitsudomi et al, 2010 Rosell et al. 2012 Van Cutsem et al. 2015
Evading growth suppressors	Cyclin dependent kinase inhibitors (Sánchez-Martínez et al. 2015)	Palbociclib	Finn et al. 2015
Resisting programmed cell death	Proapoptotic BH3 mimetics (Bcl-2 and Bcl-X_L inhibitors) (Zhang et al. 2007)	GX015-070 TW-37 ABT-737	Li et al., 2007; Perez- Galan et al. 2007; Hwang et al. 2010 Mohammad et al 2007; Wang et al. 2008 Del Gaizo Moore et al. 2007; Deng et al. 2007; van Delft et al. 2006
Enabling replicative immortality,	Telomerase inhibitors	GRN163L	Herbert et al 2005; Roth et al. 2010
Inducing angiogenesis,	Inhibitors of VEGF signaling (El-Kenawi and El-Remessy. 2013)	Bevacizumab Vatalanib Sunitinib	Reck et al. 2009; Strickler and Hurwitz. 2012 Dragovich et al. 2014 Socinski et al. 2008; Novello et al. 2009
Tissue invasion and metastasis	Inhibitors of HGF/c-Met (Blumenschein et al. 2012)	Rilotumumab Cabozantinib Foretinib Tivantinib	Schoffski et al. 2011 Kurzrock et al. 2011 Seiwert et al. 2009 Santoro et al. 2010

Table 1 Drugs that interfere with the acquired capabilities necessary for tumour growth and their molecular mechanisms

1.6 Head and neck squamous cell cancer (HNSCC)

For the purpose of this study, head and neck squamous cell cancer (HNSCC) refers to squamous cell carcinoma involving the oral cavity (tongue, floor of mouth, buccal mucosa, lower alveolus, upper alveolus and hard palate) and pharynx (Figure 6).

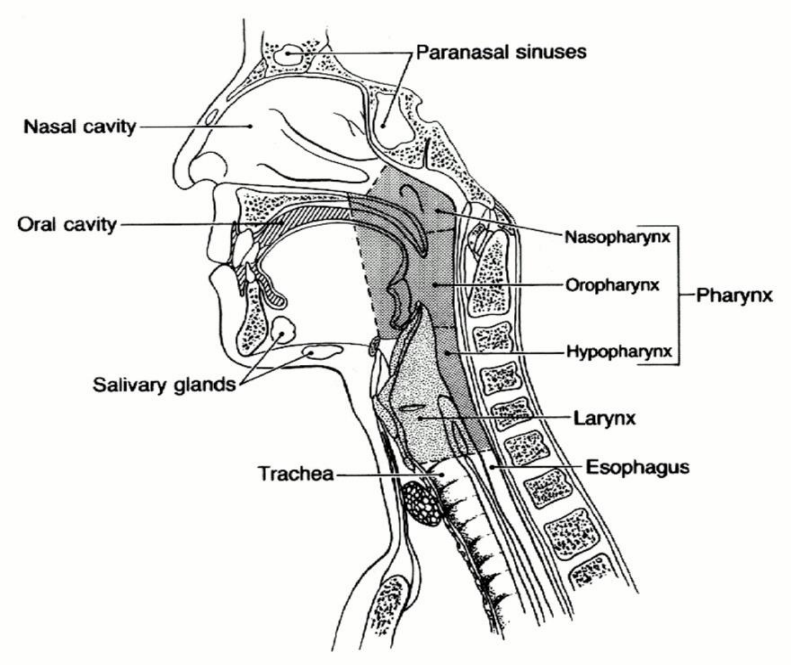


Figure 6 Anatomy of the upper aero-digestive tract (Holland – Frei manual of cancer medicine 2005)

The overwhelming majority of head and neck cancers are squamous cell carcinoma which account for more than 90% of malignant tumours of the oral cavity (Daley and Darling 2003). HNSCC accounts for approximately 2% of all carcinomas in the UK (UK cancer statistics – Cancer Research UK 2015) and worldwide, oral and pharyngeal carcinoma grouped together is the 6th commonest cancer, with two thirds of the cases arising in developing countries (Warnakulasuriya 2009). In most

countries, the five-year survival rates for cancers of the tongue, oral cavity and oropharynx are around 50% (Warnasurakuriya 2009).

1.6.1 Aetiology

The most important aetiological factors for head and neck cancers include excessive intake of tobacco (either by smoking or chewing), and alcohol, either as independent risk factors, or as a synergistic effect (Decker and Goldstein 1982; Maier et al. 1992). The risk for development of oral cancer is 3 to 9 times greater in those who smoke or drink and as much as 100 times greater in those who both smoke and drink heavily than in those who neither smoke nor drink (Mao et al. 2004; Neville and Day 2002). Human papilloma virus has also become a major etiological factor in a subset of HNSCC (Lopes et al. 2011). Other risk factors implicated include poor hygiene in the oral cavity, environmental contaminants such as paint fumes, dietary factors and use of marijuana (Petti 2009).

1.6.2 Diagnosis/ staging

The gold standard for diagnosis of head and neck cancer is biopsy of a clinically suspicious area and histological analysis of the specimen. It is graded histologically as well differentiated, moderately differentiated or poorly differentiated carcinoma.

Head and neck tumours are staged by the UICC: TNM of malignant tumours (UICC -International Union Against Cancer; TMN – Tumour / Node / Metastasis). This classification describes the anatomical extent of the disease based on an assessment of the following categories: the extent of the primary tumour (T), the absence or presence and extent of regional lymph node involvement (N) and absence or presence of distant metastatic spread (M). The categories are grouped together as a stage grouping – I, II, III, IV, V (Fleming et al.1997; Sobin et al. 2009).

Stages I and II denote early stage disease and stages III – V late stage disease. This staging system helps to guide treatment selection in clinical practice, and it has been shown that survival is strongly associated with TNM stage.

1.6.3 Molecular epidemiology

Like most epithelial cancers, HNSCC develops through the accumulation of multiple genetic and epigenetic alterations in a multistep process. Many of these alterations occur early and have been identified in premalignant lesions of the head and neck (Mao et al. 2004). The most common of all the genetic changes is loss of chromosomal region 9p21 which is an early event (Van der riet et al. 1994). Mao et al (1996) also showed alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions. The p53 tumour suppressor gene has also been shown to be the most frequently mutated gene in HNSCC (Brennan et al. 1995). Gene amplification (cyclin D1) and protein overexpression (EGFR – epidermal growth factor receptor) have also been found (Izzo et al. 2003; Pomerantz and Grandis 2003).

Reactivation of telomerase is another early event in the tumourigenic process of HNSCC (Mao et al. 1996; Mutirangura et al. 1996), which prolongs the survival of cells with genetic abnormalities making accumulation of multiple genetic alterations possible. These genetic alterations may be used as potential tumour markers for the detection of cancer cells in clinical samples such as saliva. It has been suggested that early genetic changes may not necessarily correlate with observed cellular changes (Rosin et al. 2000; Partridge et al, 2000). This implies that cells may appear normal on histological analysis but may have undergone molecular changes. Califano et al (1996) delineated a model of molecular progression of HNSCC from

preneoplastic lesions to invasive cancer by analysing head and neck lesions at various stages of tumourigenesis to detect deletions of multiple chromosomal regions. They showed that molecular change may be present in areas of premalignant change before cellular phenotypic changes become detectable. Spafford et al (2001) demonstrated that molecular analysis of cells collected by rinsing the mouth showed the same changes as were present in tumour biopsy specimens.

1.6.4 Treatment of head and neck squamous cell cancer

The mainstay of treatment of early stage HNSCC is surgery and radiotherapy, or radiotherapy alone (Belcher et al. 2014). Systemic chemotherapy has traditionally been used as palliative therapy for recurrent/advanced stage (III and IV) head and neck cancer, but randomized trials have demonstrated an improvement in disease control rates using a combination of chemotherapy and radiotherapy, rather than either method alone (Zhang et al. 2013). One of the aims of treatment of HNSCC is preservation of organ function and this has been the goal of surgical innovation in the last decade. The use of combined modality treatment regimens (such as surgery and radiotherapy/chemotherapy) helps to preserve crucial organs such as the larynx and the tongue. The type of treatment offered depends on the stage of the disease based on the TMN classification. Advancement in understanding molecular mechanisms have led to development of therapies that specifically target the molecular pathways commonly dysregulated in Head and Neck cancers (for example overexpression of EGFR). An example of this is Cetuximab, (a monoclonal antibody which targets EGFR), which has been shown to improve overall survival when

combined with other chemotherapeutic agents or radiotherpay (Vermorken et al. 2008; Bonner et al. 2006).

1.7 The telomere / telomerase complex

The structures at the ends of eukaryotic chromosomes are termed telomeres. Telomeres are DNA – protein structures composed of tracts of repetitive hexanucleotide sequences (5'-TTAGGG-3' termed "G – rich strand" and 5'-CCCTAA-3' termed "C – rich strand", for human telomeres). Telomeres protect chromosomes from degradation and loss of essential genes, and allow the cell to distinguish between double strand breaks and natural chromosome ends (Shay and Wright 2006). In mammalian cells, the G – rich strand is longer by several hundred nucleotides resulting in a long 3' single strand overhang (figure 7). Two structures of the 3' telomeric overhang have been proposed. Ultrastructural evidence *in vitro* suggests the 3' overhang folds back on itself to form a duplex loop structure termed the T loop (Doksani et al. 2013). The other proposes the formation of dimers and tetramers involving G-quadruplexes (Palm and de Lange. 2008; Lipps and Rhodes. 2009).

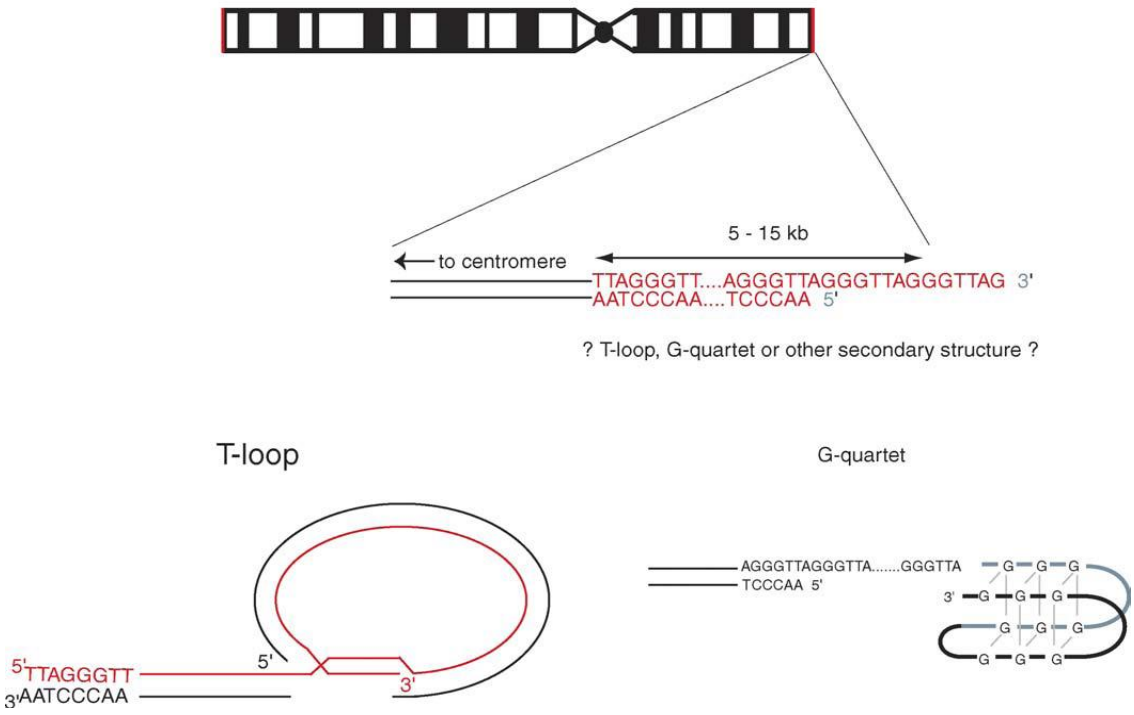


Figure 7 Telomeres - the telomeres consist of a variable number of tandem repeats of TTAGGG sequence and are therefore of variable length. The G – rich strand at the 3' end of the telomere forms a single stranded extension that can fold back on itself *in vitro* to form a T loop or G quartet structure. (Olaussen et al., 2006).

Linear DNA molecules, such as eukaryotic chromosomal DNA are unable to replicate their telomeric ends and require mechanisms besides the conventional DNA polymerases to complete the replication of their ends. With each successive round of cell division, erosion of the telomere occurs with loss of 50-200 bases of telomeric DNA (figure 8). The telomeres eventually become so short that they can no longer effectively protect the ends of chromosomes, at which point the cell goes into crisis which is characterized by chromosome fusion and widespread apoptotic death (this phenomenon was first described by McClintock in 1941). The shortened telomeres are recognized as DNA damage which form a telomere dysfunction

induced focus (TIF) and typically, cells respond to TIFs through senescence or apoptosis (Sekaran et al. 2014)

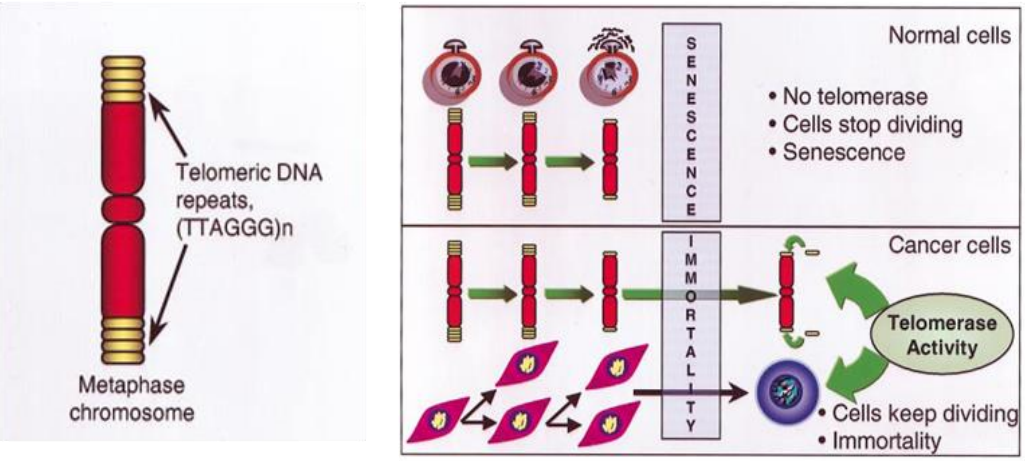


Figure 8 Telomere structure – regulation of telomere length in normal and cancer cells by telomerase (Keith et al 2001)

The proteins associated with telomeres are called the ‘shelterin complex’ which consists of 3 core subunits TRF1, TRF2 and POT1, and three additional proteins, TIN2, TPP1, RAP1. These proteins form a complex that enable DNA damage response surveillance machinery to distinguish telomere DNA from sites of genomic DNA damage. Some telomere associated proteins bind directly to the telomeric repeats (T2AG3), whilst others are associated with the telomere via protein-protein interactions (figure 9). TRF 1 and TRF 2 (telomeric repeat binding factors 1 and 2) bind double stranded telomere sequences and POT 1 (protection of telomeres 1) associates with the 3’ single stranded overhang. TRF 1, TRF 2, and POT 1 interact together and associate directly or indirectly with other proteins such as Tankyrase 1 and 2 and TIN 2 - TRF1 interacting protein 2 (Jafri et al. 2016). The telomeric DNA and telomere associated proteins play an essential role in stabilizing the

chromosome ends. The protective function of the telomere is mediated by activities of the telomere-associated protein complexes (Lu et al. 2013).

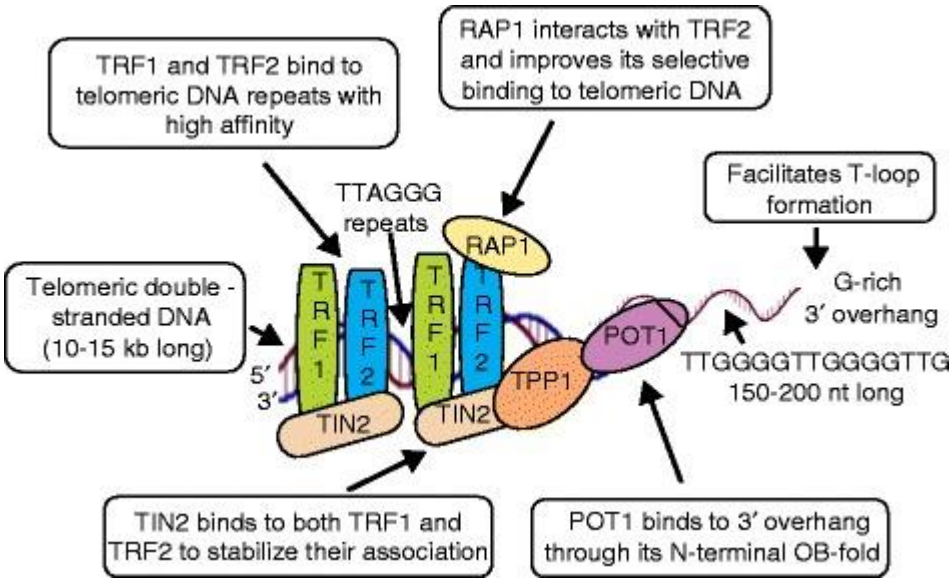


Figure 9 Schematic representation of telomeric DNA and components of the shelterin complex (Jafri et al. 2016)

Telomerase is the enzyme responsible for telomere regeneration. It is a ribo–nucleoprotein enzyme responsible for adding telomeric repeats onto the 3’ ends of chromosomes (Greider and Blackburn 1985). It is composed of two essential components: a protein subunit TERT – telomerase reverse transcriptase termed hTERT in humans (Nakamura et al 1997), and an RNA component – telomerase RNA (hTR in humans). Telomerase uses its integral RNA component as a template to synthesize telomeric DNA directly onto the ends of chromosomes (Feng et al 1995). Human telomerase is regulated during development and differentiation, mostly through transcriptional control of hTERT. hTR is ubiquitously expressed in mammalian cells (Feng et al. 1995), but the expression of hTERT is restricted to cells that exhibit telomerase activity. This indicates that hTERT is the rate-limiting component of the telomerase enzyme (Xi and Cech. 2014).

1.8 Telomerase and cancer

As previously described, telomeric repeats help maintain chromosomal integrity but linear eukaryotic chromosomes are unable to replicate their telomeric ends. In most normal human cells, telomeric DNA is progressively lost with each round of cell division resulting in the shortening of telomeres to critical length with loss of the ability of telomeres to protect the ends of chromosomal DNA and subsequent widespread chromosomal fusion and degradation (Blasco et al 1997; Counter et al 1992). Normal somatic cells exhibit a limited number of replicative cycles in culture and this limitation has been associated with two barriers of proliferation (i) senescence, a non-proliferative but viable state and (ii) crises leading to cell death. In the early 1960's Leonard Hayflick showed that normal human fibroblasts would stop growing after a certain apparently predetermined number of divisions and enter into a state known as replicative senescence (Hayflick and Moorhead 1961). The limited replicative life span of human cells has been postulated to serve as an important barrier to malignant transformation (Sager R 1991; Hahn et al 1999) and the ability to bypass replicative senescence is thought to be one critical rate-limiting step in the evolution of most malignancies. As previously described, cancer cells need to acquire genetic mutations that override the normal mechanisms controlling cellular proliferation in order to acquire the ability to multiply indefinitely and therefore form malignant tumours.

Telomerase is the enzyme that provides cancer cells with unlimited replicative capacity. It is a specialized enzyme first identified in ciliates by Greider and Blackburn in 1985, and it prevents telomeric DNA from being shortened during cell

division. In most normal cell types, telomerase activity is down regulated; in cancer cells, however, telomerase expression is activated. Telomerase has been found to be over expressed in 85-90% of cancer cells such as hepatocellular carcinoma, colon cancer, Wilm's tumour, breast cancer, prostate cancer, brain tumours and head and neck squamous cell carcinoma (Kim et al 1994; Shay and Baccetti 1997). Telomerase activity is absent in about 15% of malignant tissues and in this situation, the tumours maintain the length of telomeres by a non-telomerase based mechanism known as alternative lengthening of telomeres (ALT) which also permits unlimited replication of cells (Henson et al 2002; Dilley and Greenberg. 2015). Majority of somatic cells and benign cells do not express the telomerase activity, but the following normal tissues have been found to have increased telomerase activity: germ line tissues (Kim et al 1994); haematopoietic stem cells (Broccoli et al 1995) and keratinocytes (Harle-Bachor and Boukamp 1996).

The significance of telomerase in malignant transformation was demonstrated by Hahn et al (1999) where they were able to create human tumour 'like' cells from normal cells via the ectopic expression of the catalytic subunit of telomerase. The presence of telomerase activity has also been correlated with a resistance to induction of both senescence and crises; conversely, suppression of telomerase activity leads to telomere shortening and to the activation of one or the other proliferative barriers (Hanahan and Wienberg. 2011). As telomerase activity has been found in almost all human cancer tissues and cancer cell lines, it appears to be the most universal of all known tumour markers (Kim. 1997; Shay and Bacchetti. 1997).

1.9 Detection of telomerase

A method of detecting telomerase is via the detection of telomerase activity using the telomeric repeat amplification assay (TRAP assay). Other methods of telomerase measurement include the detection of telomerase expression (as opposed to detection of telomerase activity with the TRAP assay), such as immunohistological detection of hTERT protein in cultured cells and tissue sections and detecting the expression of hTERT mRNA using methods to measure RNA expression such as northern blotting, *in situ* hybridization and RT-PCR. Further details of the methodologies used in this thesis are described below.

TRAP assay

Telomerase enzyme activity is commonly detected and quantified by the polymerase chain reaction (PCR)-based assay known as the **t**elomeric **r**epeat **a**mplification **p**rotocol (TRAP) assay. Previously, the conventional primer-extension based assay for detecting telomerase activity required large amounts of sample material and only allowed detection of telomerase with limited sensitivity. These disadvantages were overcome by the TRAP assay in which the telomerase reaction product is amplified by PCR. The amount of PCR product generated is proportional to telomerase protein expression and activity. The standard TRAP assay (described by Kim et al 1994) is a 2 staged PCR based assay. In the first stage, telomerase adds 5'- TTAGGG-3' repeats to the end of a synthetic primer that has a telomere-like sequence. In the second stage, the extended products are amplified using a reverse primer complimentary to the repeat sequences. The standard TRAP described by Kim et al requires a radioactive label and visualization of the PCR amplification products by autoradiography after gel electrophoresis.

The TRAP assay and variations on this assay are the most widely used methods of monitoring telomerase activitiy. Various other modifications have been suggested since the introduction of the TRAP assay some of which do not require radioactivity and are available as commercial kits. These include: TRAPezeXL - TRAP with Amplifluor primers which are energy transfer primers that emit fluorescence only upon incorporation into the PCR products of TRAP (Uehara et al.1999) and TRAP-ELISA (figure 9). TRAP-ELISA assay is based on the principle that a biotinylated substrate primer is used to immobilise the denatured PCR product to a streptavidin-coated microplate, hybridized with a digoxigenin-labelled probe and detected with an antibody against digoxigenin conjugated to peroxidise (Longchampt et al 2003; Yamamoto and Hirakawa 2005).

The TRAP assay is highly sensitive and specific for telomerase activity in tumour samples (Hess and Highsmith 2002), but it has certain limitations. A drawback of the TRAP assay is when it is used on tissue homogenates; the expression of telomerase activity in a low number of cells may be masked by its absence in the majority of cells in a tissue and also, information on the cell type expressing telomerase is lost.

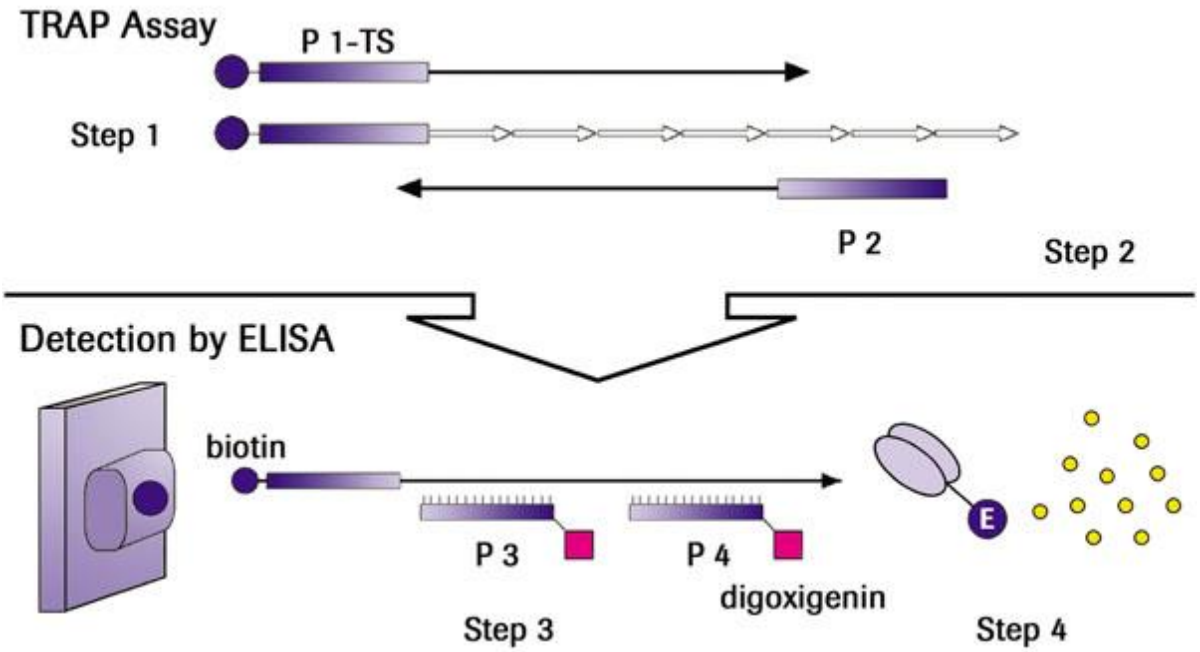


Figure 10 Principle of the TRAP ELISA. Steps 1 and 2– telomerase adds telomeric repeats to the 3' end of the biotin labelled synthetic (P1-TS) primer, and the elongation products are amplified by PCR using the primers (P1-TS and P2), generating PCR products with telomerase specific 6 nucleotide increments. Steps 3 and 4 – the PCR product is hybridized to a digoxigenin labelled telomeric repeat specific probe and this is then immobilized via the biotin labelled primer to a streptavidin-coated microplate (Roche diagnostics Shi et al 2013; Giri et al 2009).

Immunohistochemical detection of hTERT protein

The expression of hTERT protein can be identified in individual cells via immunohistochemistry in cultured cells and tissue sections (Hiyama et al 2001). The procedure was conceptualized and first implemented by Dr. Albert Coons in 1941. Immunohistochemistry exploits the principle of antibodies binding specifically to antigens in cells. The antibody-antigen interaction can be visualized in a number of ways such as the antibody conjugated to an enzyme, such as peroxidase, resulting in a colour-producing reaction. Alternatively, the antibody can also be tagged to a fluorophore, such as fluorescein or rhodamine. The TRAP assay described above is able to estimate telomerase activity but is unable to determine telomerase activity in

each cell. The primary antibody used in this study was the hTERT monoclonal antibody – the methodology will be discussed in subsequent chapters.

1.10 Telomerase activity in body fluids

Telomerase activity has been shown to occur in cytological samples (Yamamoto and Hirakawa 2005; Cunningham et al. 1998). Cunningham et al used the TRAP assay to detect telomerase expression in fine needle aspirates and fluid specimens from different body sites with different histological diagnosis. They found that 6 out of 12 fine needle aspirate specimens that were cytologically positive for malignant cells expressed telomerase activity whilst none of the cytologically atypical or negative specimens tested positive for telomerase activity. Four of the six fluid specimens that were cytologically positive and 1 of the cytologically negative fluid specimens expressed telomerase. Yamamoto and Hirakawa detected telomerase activity in 8 of 16 malignant cases (ascitic, pleural and pericardial fluid; peritoneal washings) using the TRAP assay and 10 of 16 malignant cases using RT PCR to detect hTERT mRNA expression. They found that the detection of mRNA expression of hTERT is a more useful method than TRAP assay for the detection of cancer cells in body fluids. Telomerase activity has also been detected in other body fluids such as urine (Melissourgos et al. 2003; Casadio et al. 2009). Using RTPCR, Melissourgos et al in 2003 were able to detect the expression hTERT in the urine of 92% of patients with bladder cancer whilst only 44% yielded a positive result by cytology suggesting a more sensitive method of detection. Casadio et al (2009) showed that urine telomerase activity was a good marker for early diagnosis of bladder tumours in symptomatic patients.

1.11 Telomerase and head and neck squamous cell cancer

As previously described in section 1.7, telomerase activity is down regulated in most normal cell types but has been found to be activated and over expressed in 85-90% of cancer cells (Kim et al. 1994; Shay and Wright 1996; Shay and Baccetti 1997). The fact that telomerase activity is mainly detected in cancerous cells as opposed to benign and normal cells has attracted investigations into its possible use as a prognostic and diagnostic marker. As with other cancers, telomerase has been found to be active in approximately 80% to 90% of tumour tissues of the head and neck squamous cell carcinomas whereas it is not active in normal head and neck tissues (Mao and El-Nagger 1996; Rossi et al. 2005). Furthermore, telomerase activation has been shown to be an early event in the progression of HNSCC (Mao et al. 1996). Curran et al (1998) were able to detect telomerase activity using the TRAP method in 18 of 20 oral cavity squamous cell carcinoma tissues. Expression of the telomerase catalytic protein subunit (hTERT) in HNSCC has also been investigated by immunohistochemistry. Kim et al (2001) were able to demonstrate elevated levels of hTERT expression in paraffin embedded squamous cell carcinoma biopsy specimens. Boyle et al (1994) have previously demonstrated the possibility that cancer cells may be shed into the saliva of head and neck cancer patients. They identified tumour specific mutations from exfoliated cells in saliva that had been obtained from the patients preoperatively. Using the PCR-based radioactive methods for detection, telomerase activity has been detected in oral rinses of HNSCC patients but the rate of detection and level of positivity has been low (Califano et al. 1996; Mutiranga et al. 1996; Sumida et al. 1998).

Saliva and Head and neck squamous cell cancer

Whole saliva is a mixed fluid that is derived predominantly from 3 pairs of salivary glands. It contains expectorated bronchial and nasal secretions; desquamated epithelial cells and other cellular components; and food debris. Some of these desquamated epithelial cells could be shed from cancerous tissue. Saliva is therefore one of the most reliable tools for diagnosing oral squamous cell carcinomas due to its direct contact with oral cancer lesions (Radhika et al. 2016). An ideal diagnostic test should be noninvasive, inexpensive, and easy to perform; the marker evaluated should be detected in early cancer stage and grade; the test should be highly accurate to reduce the rate of false positive and negative results. In the context of head and neck cancer, saliva would serve as an ideal substrate to investigate biological markers in this disease.

In 1998, the National Institute of Health Biomarkers Definitions Working Group defined a biomarker as ‘a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (Biomakers Definitions Working Group. 2001). Biomakers have been classified based on biomolecules into DNA biomarkers, RNA biomarkers and protein biomarkers (Mishra and Verma. 2010). The increasing use of biomarkers to assess systemic health and disease status has led to study of cancer markers in saliva.

Saliva is known to contain RNA from various sources such as secretions from salivary glands, gingival crevice fluid and desquamated epithelial cells. Saliva has been shown to contain ribonucleases (Eichel et al. 1964). It is not clear how RNA and ribonucleases can coexist in saliva, but it has been shown by Park et al (2006) that salivary RNA is protected from degradation as similarly occurs with plasma RNA

(Tsui et al. 2002). Markopoulos et al (2010), in their study on salivary biomarkers for oral cancer detection showed that the molecular diagnosis of oral squamous cell carcinoma can be explored in three ways – a) changes in cellular DNA which result in - b) altered mRNA transcripts leading to - c) altered protein levels intra or extra-celullarly. Changes in cellular DNA include presence of point mutations, deletions, translocations, amplifications and methylations, cyclin D1, epidermal growth factor receptor and microsatellite instability (Bano et al. 2015). P53 gene mutations are present in saliva samples of patients with head and neck cancers (Liao et al. 2000). Li et al (2004) using microarray analysis of the saliva transcriptone showed that mRNA levels for specific proteins are elevated in HNSCC, compared to healthy control subject. CD44, a cell surface transmembrane glycoprotein which has been found to be over-expressed in the earliest stages of carcinogenesis is detectable in body fluids and can be measured with simple, inexpensive assays (Dasari et al 2014).

1.12 The head and neck squamous cell cancer problem

HNSCC is a complex disease arising from different anatomical sites in the head and neck (as above). It is associated with severe disease and treatment related morbidities. Despite therapeutic advances in the treatment of HNSCC, the overall 5 year survival from the disease has remained unchanged over the last 30 years and is amongst the lowest of the major cancers. This is largely reflected by the fact that most cases are in advanced stages at the time of detection, despite easy accessibility for regular examination (Warnasurakuriya 2009). Several factors have been shown to be responsible for this low survival rate:

Diagnostic problems

These include

(i) Diagnostic delay due to patient factors and physician factors (from diagnostic difficulty), which result in significant proportions not diagnosed early enough and treatment commenced only when they reach an advanced stage (McGurk et al. 2005, Allison et al. 1998; Kowalski et al. 1994).

(ii) Successfully treated early stage disease patients have a high incidence of a second primary tumour cancer of the head and neck (Slaughter et al. 1953; Jang et al. 2001). However, the prognosis in early stage disease is much better than late stage disease and can be as high as 80% as it requires much less complex and aggressive treatment (Silverman Jr 1988, van der Schroeff and Baatenburg de Jong 2009).

The entire upper aero digestive tract of head and neck cancer patients is chronically exposed to the common etiological carcinogens such as tobacco smoke which therefore predisposes the patients to multiple oral precancerous lesions (Jang et al. 2001). As described previously, most head and neck cancers arise in a precancerous field of epithelial cells characterized by tumour associated genetic changes. These abnormal fields can be extensive but may not be visible at clinical examination (Tabor et al. 2001). Diagnosis is reliant on biopsy of a clinically suspicious area and histological analysis of the specimen. This requires a very experienced pathologist but variability of interpretation of findings occurs amongst pathologists. Slaughter et al (1953) proposed a phenomenon called ‘field cancerization’ - they showed that all the epithelium outside an oral squamous cell cancer was abnormal and that 11% of the patients had another cancer elsewhere. In

this situation, a patient with multiple predisposing factors has a mixture of normal cells, potentially malignant cells; malignant cells that have yet to invade and cells that have invaded all occurring together. Thus, there can be a considerable discrepancy between the clinician's view of where the lesion is and the real extent of the lesion. When the biopsy is therefore performed, site selection is critical as there may be variability in non-uniform lesions and areas with less severe cellular change, if sampled, may be interpreted as representative of the lesion as a whole (Epstein et al. 2002). This variability may result in erroneous tissue sampling for histological diagnosis. In order to combat this and to assess areas of precancerous changes, multiple biopsies would need to be taken repeatedly and randomly for analysis which would be impractical. It has also been shown that histological grading can be subjective and shows limitations in predicting the risk of malignant progression in individual patients (Holmstrup et al. 2007). Based on this, there is thus a need for an aid to the routine histological method of diagnosis. This would be based on molecular characteristics of the tumour, be independent of the subjective histological approach to diagnosis and play a role in improving mortality and survival of these groups of patients. Precancerous fields are more promising targets for screening than early stage tumours, as the time to malignant progression is usually measured in years instead of months (Graveland et al. 2013).

Treatment problems

Systemic chemotherapy has traditionally been used as palliative therapy for advanced stage head and neck cancer and recurrent disease. The management of early stage disease is mainly with surgery and radiotherapy with the resultant issues around the morbidity (functional disability) associated with surgery which includes loss of swallow, speech and airway. Organ preservation is the goal of treatment in

order to reduce the morbidity of the disease and this has been greatly enhanced by the use of combination therapy (surgery and radiotherapy). The overall 5 year survival of the disease however remains low. There is therefore a need for better therapies which can be combined with the established modes of therapy to treat this disease and improve survival.

In order to combat the problems associated with the diagnosis and treatment of HNSCC patients, two approaches can be employed. First, a molecular based approach to the detection of the disease could aid early diagnosis in this group of patients. The detection of telomerase activity in saliva of head and neck squamous cell cancer patients using a non radioactive PCR based method could serve as a simple, non-invasive technique to aid diagnosis. Used in conjunction with histological analysis, it could serve as a useful adjunct to diagnosis and treatment providing a less invasive and efficient diagnostic medium. It may be used as a molecular marker, helping to reduce the morbidity and mortality currently associated with the disease and to improve the prognosis. Secondly, the development of novel agents that specifically target the biochemistry cancer cells, particularly those pathways contributing to the hallmarks of cancer may help to improve survival in head and neck cancer patients and survival in cancer patients as a whole.

1.13 Telomerase inhibition

The telomere/telomerase complex offers a therapeutic window in which cancer cells can be efficiently targeted by inhibitors. Telomerase has been a prime target for the development of effective therapeutics against cancer as it is expressed inmajority of cancer types (Jafri et al. 2016). The main objective of anti-telomerase therapeutics is

to selectively induce apoptosis and cell death in cancer cells while minimizing the effects on normal cells (Buseman et al. 2012).

The following section describes some of the approaches used to target telomerase including the key evidence demonstrating that telomerase is a validated target.

Validation of telomerase as a cancer inhibitory target

Telomerase has been demonstrated as one of the key enzymes for human cells to acquire immortality (Kim et al 1994). This has been confirmed in the following ways:

1) Stabilization of mortal human cells through ectopic expression of telomerase which facilitates immortalisation - a trait recognised as a crucial step during cell transformation (Bodnar et al. 1998; Vaziri and Benchimol. 1998).

2) Conversion of human fibroblasts or epithelial cells to transformed cancer cells which is facilitated by hTERT expression in conjunction with oncogenes (Hahn et al. 1999).

3) Inhibition of telomerase in immortal human cancer cell lines leads to apoptosis or senescence (Hahn et al. 1999; Herbert et al. 1999; Zhang et al. 1999; Damm et al. 2001).

Another indication that telomerase is essential for cancer progression has been shown in stage ‘4s’ neuroblastoma tumours. Neuroblastoma is a type of childhood cancer derived from specialised cells. Neuroblastoma 4s is a ‘special’ stage of disseminated neurofibroma found in children less than 1 year. Its hallmark is the possibility of spontaneous regression despite large tumour burden

(Piefer et al. 2015). Hiyama et al in 1995 studied the regulation of telomere length and activity of telomerase in neuroblastoma tumour samples. They found that most of the tumours that exhibited high telomerase activity were associated with poor prognosis, whereas most of the tumour samples from 4s neuroblastoma had low telomerase activity or short telomeres. This suggested that the low telomerase activity may be one of the mechanisms responsible for spontaneous regression in stage 4s neuroblastoma (Hiyama et al. 1995, Brodeur and Bagatell 2014; Piefer et al. 2015).

1.14 Telomerase inhibitors

As previously described, one of the most crucial differences between cancer cells and normal cells is that cancer cells have unlimited proliferative capability which they acquire via several mechanisms including the activation of telomerase. The potential therefore exists for cancer cells to be more critically affected than normal cells by any compound that interferes with the telomere/telomerase complex. This provides a level of specificity and selectivity. In the early 2000, the telomeres/telomerase complex emerged as an attractive possibility for intervention with molecular cancer therapeutics and offers multiple possibilities for the development of inhibitors. Several strategies for targeting telomere maintenance and telomerase positive cancer cells have been explored over the years. The various approaches have been reviewed by Sekaran et al (2014) - figure 11, and summarised in table 2.

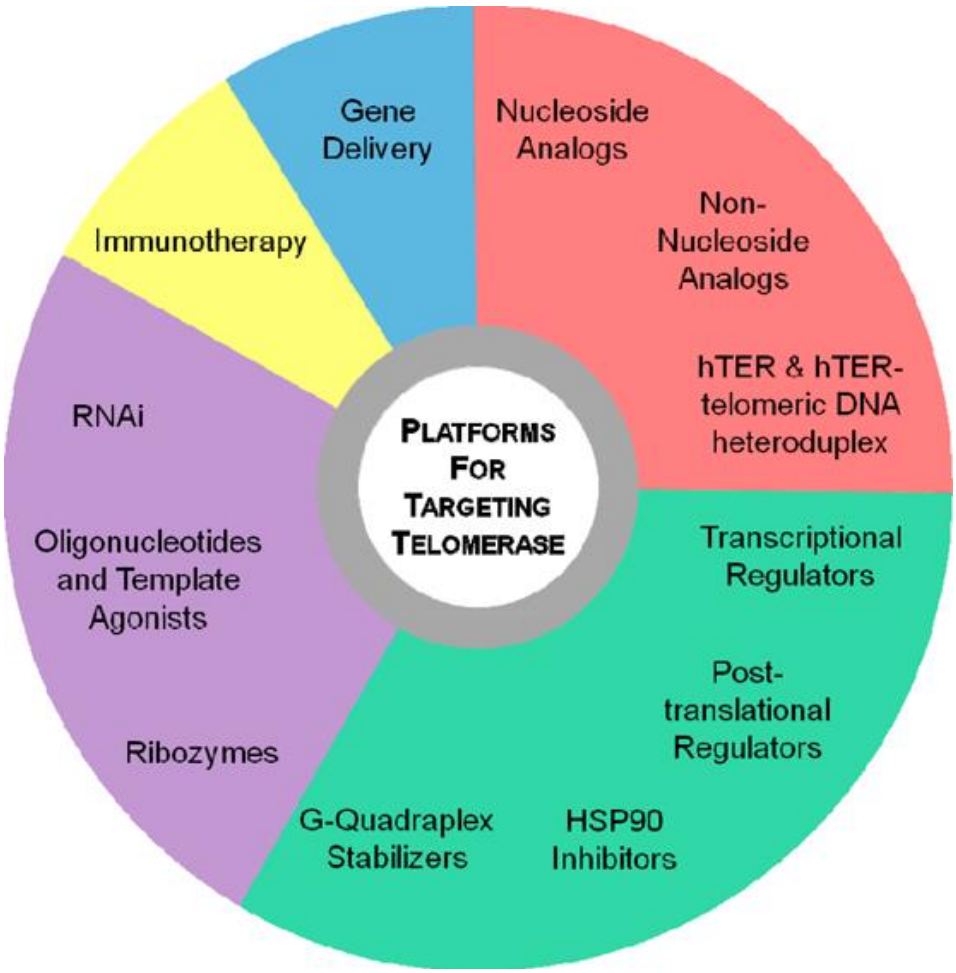


Figure 11 Targeting the overexpression of telomerase in cancer cells (Sekaran et al. 2014)

Platform	Agent	References
Nucleoside analogue reverse transcriptase inhibitor	AZT (azidothymidine)	Strahl and Blackburn 1996; Melana et al. 1998
Non-Nucleoside analogues	BIBR1532	Pascolo et al. 2002
hTERT and hTERT-telomeric DNA heteroduplex	Aminoglycosides	Dominick et al. 2004 Kaiser et al. 2006
Transcriptional regulators	TMPyP4	Grand et al. 2002
Post-translational Regulators	Protein kinase C inhibitors Tyrosine kinase inhibitors (Imatinib mesylate)	Yu et al. 2001 Uziel et al. 2005
HSP90 Inhibitors	Geldanamycin	Villa et al. 2003
G Quadruplex Stabilizers	Telomestatin	Tauchi et al. 2003
Ribozymes	Hammerhead ribozymes	Lu et al. 2011
Oligonucleotides and Template Agonists	GRN163L	Asai et al. 2003 ; Dikmen et al. 2008 ; Goldblatt et al. 2009
RNAi	siRNA therapies	Li et al. 2005; Noreen et al. 2009
Immunotherapy	hTERT derived peptides	Liu et al. 2010; Hunger et al. 2011
Gene Delivery	Telomelysin	Nemunaitis et al. 2010

Table 2 Platforms for targeting the overexpression of telomerase in cancer cells (Sekaran et al. 2014)

The first telomerase inhibitors reported were synthetic oligonucleotides which has resulted in the lead compound GRN163L (Imetelstat).This is the first telomerase inhibitor to enter clinical trials (Roth et al. 2010). GRN163L has been reported to inhibit cellular growth in cancer cell lines of diverse origins, including multiple myeloma and tumours of the bladder, breast, liver, lung and stomach (Gryaznov et al. 2007). Studies in mice carrying liver cancer xenografts showed that GRN163L alone inhibited tumour growth, in addition to sensitizing the tumour cells to conventional chemotherapy (Djojosubroto et al. 2005). Phase I trials of GRN163L in patients with chronic lymphocytic leukaemia, multiple myeloma and solid tumours such breast cancer and non-small cell lung cancer have been completed (Bruedigam and Lane 2016). Phase II trials to evaluate GRN163L in a maintenance setting following treatment with bevacizumab in advanced non-small-cell lung cancer (Chiappori et al. 2015) failed to improve progression free survival in this group of patients. Currently, GRN163L is being tested in two clinical trials both currently in phase 2: IMbark trial in Myelofibrosis patients, and IMerge trial in Myelodysplastic syndrome. Both studies commenced in 2015, patients are currently being enrolled in the IMerge trial and in the IMbark trial, enrolment of patients has closed, but participants are currently receiving treatment with monitoring.G quadruplex stabilizers.

The compounds used for experiments in this thesis are designed to be G – quadruplex stabilizing ligands, therefore, this will be the focus of the thesis. These agents are directed at the telomere (Hurley et al. 2000). As mentioned previously, the G-rich single stranded telomere overhang has been shown to fold back on itself *in vitro* forming T-loop or G quadruplex structures (Doksani et al. 2013; Palm and de Lange. 2008; Lipps and Rhodes. 2009). In order for telomeric DNA extension to

occur, telomerase must hybridize to its DNA primer which it is unable to do in the presence of G - quadruplex formation. These G quadruplex structures are poor substrates for telomerase, and agents that specifically target these structures are termed G-quadruplex stabilizing agents. Zahler et al (1991) demonstrated that increasing the stability of G quadruplex structures at telomeres via small molecules can negatively disrupt telomere structure and also inhibit telomerase activity (Haider et al. 2003; Patel et al. 2007). These agents stabilise the G–quadruplex structures by intercalation and therefore block telomerase during the elongation step. G-quadruplex stabilizing agents belong to different molecular groups such as porphyrins, acridine, anthraquinones and fluorenone based compounds. The first G-quadruplex ligands explored as telomerase inhibitors were disubstituted anthraquinone derivatives (Perry et al. 1999). Other examples include BRACO-19 (9-[4-(N, N-dimethylaminophenylamino]-3,6-bis(3-pyrrolodinopropionamido acridine) (Gowan et al. 2002; Burger et al. 2005) and RHPS4 (3,11 –difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl] acridinium methosulphate) (Leonetti et al. 2004; Cookson et al. 2005). The G-quadruplex ligand, quarfloxin - a fluoroquinolone derivative, has been shown to induce apoptosis in adenocarcinoma cells (Drygin et al. 2009). One of the hurdles of G – quadruplex targeting has been the poor pharmacokinetic and poor pharmacodynamic properties of promising compounds, there are questions about safety as they may affect the telomere structure of normal cells as well as numerous G-rich regions of the genome (Gunaratnam et al.2007; Patel et al.2007).

1.15 Project rationale and aims

Head and neck squamous cell cancer is a disease with high morbidity and mortality especially when detected late as frequently happens. Survival rates have remained unchanged in the past three decades. Patients with HNSCC are also at increased risk of developing a second primary tumour. A simple non-invasive technique of screening and detection would be beneficial in early disease in new patients and in patients with a previous history of the disease. This could help reduce the high mortality associated with this disease.

The purpose of this study was firstly to investigate the feasibility of detection of telomerase activity in the saliva of patients diagnosed with upper aero-digestive tract squamous cell carcinoma using a nonradioactive telomerase PCR-ELISA method. This could serve as a diagnostic marker of the disease. Tissues were also collected from the same patients in order to test the presence or absence of telomerase using immunohistochemical methods. In order to do this, ethics approval was sought from the local research and ethics committee. Patients diagnosed with HNSCC were then identified, and saliva and tissue samples collected after consents were obtained. Saliva samples were also collected from age and sex matched healthy controls. The samples were then investigated for telomerase activity.

As Telomerase has been validated as a target for cancer cell inhibition, the second aim of this study was to screen a range of novel compounds with a view to identifying effective telomerase inhibitors. Such compounds could offer potential therapeutic options which could improve the survival of head and neck cancer patients. In order to do this, telomerase activity was first of all demonstrated in a range of cancer cell lines and a cell line with high telomerase activity was selected

for further study. The potential of the compounds as telomerase inhibitors was investigated by determining the effect of these compounds on the telomerase activity in cell lysates. The cytotoxic effects of the selected compounds were then investigated using telomerase positive cell lines.

2 MATERIAL AND METHODS

The following section describes the materials and methods used for the detection of telomerase study, and the inhibition of telomerase study.

2.1 General materials

2.1.1 Reagents and disposables

All chemicals were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) and all disposable plastics were obtained from Corning Incorporated Costar (Schipol - Rijk, Netherlands), unless otherwise stated.

2.1.2 Cells lines

All cell lines (with the exception of HUVEC cells) were originally purchased from American Tissue Culture Collection (ATCC, UK) or from European Collection of Cell Cultures (ECACC, UK) and are of human origin. Human umbilical vein endothelial cells (HUVEC) were kind gifts from Dr Kirstie Eccles (University of Bradford, UK). Table 3 shows the cells and their origin.

Cell lines	Pathological origin	Reference
A549	Human lung adenocarcinoma	Giard et al. 1973
H157	Human oral squamous cell carcinoma	Prime et al. 1990
H460	Human lung adenocarcinoma	Carney et al. 1981
EJ138	Human bladder carcinoma	O'Toole et al. 1983
HT29	Human colorectal adenocarcinoma	Fogh and Orfeo 1977
A2780	Human ovarian carcinoma	Hamilton et al. 1984
HT1080	Human fibrosarcoma	Rasheed et al. 1974
PC3	Human prostate cancer cell line	Kaighn et al. Invest Urol 1979

Table 3 Cell lines used in study.

2.1.3 Kits

Telomerase PCR-ELISA kit was purchased from Roche Diagnostics, Mannheim, Germany. RNAqueous®-Micro and RETROscript® Kit were from Ambion – Applied Biosystems, Warrington, UK. Taq PCR Core Kit was from Qiagen, Crawley, UK.

DakoCytomation LSAB 2 System-HRP® kit was purchased from DakoCytomation, Glostrup, Denmark. Vectastain ABC kit and DAB peroxidase substrate kit were purchased from Vector Laboratories LTD, Peterborough, UK.

Senescence β – galactosidase staining kit was purchased from New England Biolabs (UK) Ltd, Cell Signalling Technology

2.2 Materials used in the detection of telomerase study

2.2.1 Patient and Healthy volunteer data

The information provided in tables 4 and 5 is simply a list of the samples obtained. Details of sample collection, ethical approval and processing of samples can be found in section 3.

Patient number/Sex/Age (yr)	Tumour site	Tumour stage
1 / F / 35	Soft palate	T1
2 / M /44	Posterior tongue	T2
3 / M / 56	Soft palate	T2
4 / F / 63	Lateral tongue	T1
5 / M / 51	Posterior tongue	T2
6 / M / 59	Lateral tongue	T1
7 / F / 57	Base of tongue	T4
8 / M / 82	Lateral tongue	T2
9 / M / 70	Soft palate	T2
10 / M / 67	Lateral tongue	T2
11 / M / 45	Anterior tongue	T4
12 / M / 62	Soft palate	T4
13 / M / 69	Floor of mouth	T3
14 / M / 48	Lateral tongue	T1

Table 4 Patient details of 14 patients with squamous cell carcinoma of the oral cavity

Healthy Control number/Sex/Age (yr)
1 / F / 36
2 / M /46
3 / M / 57
4 / F / 64
5 / M / 51
6 / M / 59
7 / F / 59
8 / M / 82
9 / M / 70
10 / M / 61
11 / M / 48
12 / M / 63
13 / M / 70

Table 5 Healthy volunteers

2.2.2 Fluids for mouth wash

Hartmann's solution and sodium chloride were obtained from Baxter Healthcare, UK.

2.2.3 Immunohistochemistry materials

8-well chamber microscopic slide were obtained from Fisher Scientific UK Ltd. Twin frosted glass microscope slides were from VWR International Ltd, Poole, UK. Goat serum was from Invitrogen, Gibco, NY. Primary antibody (hTERT rabbit monoclonal antibody) was from Epitomics, CA, USA. Secondary antibody (Biotinylated Anti-Rabbit IgG, made in goat) was from Vector Laboratories Ltd, Peterborough, UK.

TBST (Tris-buffered saline + Tween 20) was from Sigma-Aldrich Company Ltd, Poole, Dorset, UK).

2.3 Materials used in inhibition of telomerase study

2.3.1 Drugs

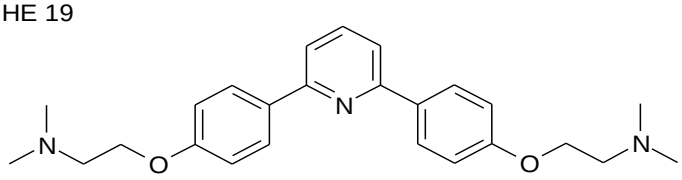
Berberine chloride dihydrate, cisplatin, doxorubicin hydrochloride and 5-fluorouracil were obtained from Sigma (Poole, Dorset, UK). Berberine chloride was dissolved in water. Cisplatin, doxorubicin and 5-fluorouracil were dissolved in DMSO.

2.3.2 Synthetic compounds

These compounds were provided by Dr Richard Wheelhouse, School of Pharmacy, University of Bradford, UK. The compounds were dissolved in DMSO or sterile water. Stock solutions of 1mM or 5mM were prepared and from these, serial dilutions were prepared to a varying range of concentrations. The stock solution was stored at - 20^{o}C. The compounds were designed to target DNA quadruplexes (Wheelhouse et al, 2006), and therefore act as inhibitors of the telomere – telomerase complex.

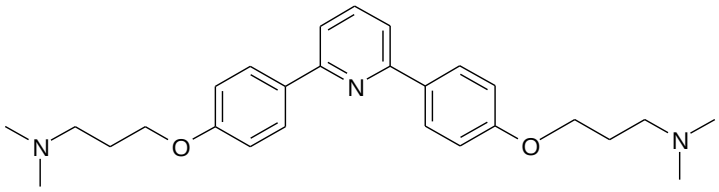
34 compounds in all were screened for telomerase inhibition (appendix V and VI). The structures of the final 4 compounds selected for further analysis are shown in figure 12 below.

HE 19



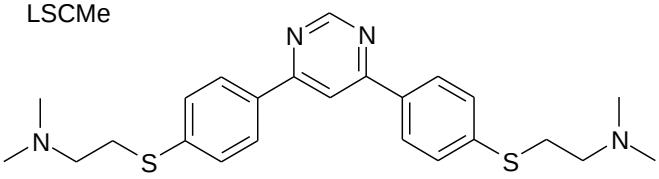
2,6 - Bis - [4' - (3'' - dimethylamino) ethoxy] phenyl] pyridine

HE 20



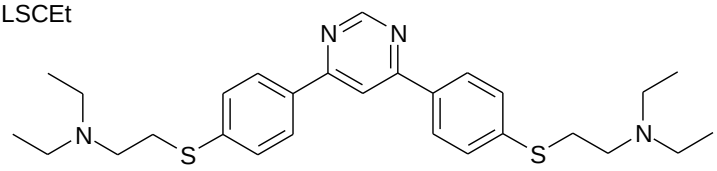
2,6 - Bis - [4' - (3'' - dimethylamino) propoxy] phenyl] pyridine

LSCMe



4,6 - Bis - [4' - [[2'' - (dimethylamino) ethyl] mercapto] phenyl] pyrimidine

LSCEt



4,6 - Bis - [4' - [[2'' (diethylamino) ethyl] mercapto] phenyl] pyrimidine

Figure 12 Structure of novel compounds. The 4 compounds shown above were selected out of 34 compounds screened for further analysis.

2.4 Methods

2.5 Methods common to the detection of telomerase and the inhibition of telomerase studies

2.5.1 Culture and medium conditions

A549, H460, EJ138, HT29, A2780 and HT1080 cells were grown in complete RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2mM L-Glutamine and 1mM sodium pyruvate. H157 cells were incubated in DMEM: HAMS F12 (1:1) supplemented with L-glutamine and sodium hydrocortisone succinate (0.5 µg/ml). HUVEC cells were incubated in M199-M2154 supplemented with 20% human serum, penicillin/streptomycin (100U/100mg/ml) fungizone (2.5µg/ml) and L-glutamine. All cells were incubated at 37C in a CO_2 enriched atmosphere (5%).

2.5.2 Cell line maintenance

Cells were routinely maintained as a monolayer culture in T75 flasks and the medium (10 ml) was replaced every 3 to 4 days. The cells were passaged when they attained approximately 70 to 80% confluence as determined by visual inspection. The growth medium was removed, and the cells were washed twice in Hanks balanced salt solution (HBSS). The cells were then incubated at 37C in 2ml of 0.25% Trypsin-EDTA for 5 min (or until the cells became detached). Complete media (10 ml) was added and the cell suspension centrifuged for 5 min at 1000*g* at room temperature. The supernatant was discarded, and the cells were re-suspended in complete media then re-seeded into new flasks containing fresh medium. Cell were discarded after 10 passages and replaced with fresh stocks.

2.5.3 Cell counting

Cells were trypsinised using 0.25% Trypsin-EDTA and re-suspended in fresh medium as described above. 10µl of the cell suspension was placed in each Neubauer haemocytometer chamber. Cell counts were taken from a total of ten grids, 5 counts from each of the haemocytometer chambers using a low power objective (x10). The cell concentration was expressed as (mean cell count) x 10^4 cells per ml of cell suspension.

2.5.4 Determination of telomerase activity

Telomerase activity was assayed using the TeloTAGGG Telomerase PCR ELISA kit. The TeloTAGGG Telomerase PCR ELISA is a photometric enzyme immunoassay which utilizes the telomeric repeat amplification protocol (TRAP) for the detection of telomerase activity. It is an extension of the original method described by Kim et al (1994). The assay is based on the principle that telomerase in the cell extract adds telomeric repeats to biotin labeled synthetic primers which is amplified by PCR. This generates PCR products with telomerase specific 6 nucleotide increments. An aliquot of the PCR product is denatured and hybridized to a digoxigenin (DIG) labeled, telomeric repeat specific detection probe. The resulting product is immobilized via the biotin labeled primer to a streptavidin-coated microplate. The immobilized PCR product is then detected with an antibody against digoxigenin (anti-DIG-POD) that is conjugated to peroxidase. A peroxidase substrate (tetramethyl benzidine) is added, which is metabolized by peroxidase to form a coloured reaction product. A stop reagent (containing <5% sulfuric acid) is added and the absorbance is measured with a microplate (ELISA) reader. Detailed methodology is described below.

Preparation of cell extracts

According to the manufacturer's instructions, $2x10^5$ cells in suspension were used to prepare cell extracts. The cells were put into a 0.5ml eppendorf tube (Thistle, UK) and centrifuged at 3000*g* for 10 min in a refrigerated centrifuge at 4^0C. The supernatant was removed, and the cells were re-suspended in phosphate buffered saline (PBS) centrifuged at 3000*g* for 10 min in refrigerated centrifuge at 4^0C. This step was repeated. The cells were then re-suspended in 200µl of lysis reagent (provided in the kit), incubated on ice for 30 min, then centrifuged at 16000*g* for 20 min at 4^0C. The supernatant was carefully removed and transferred into a fresh 0.5ml eppendorf tube. The cell extract was stored at -80^0C until required or used to perform the TRAP reaction immediately.

Telomeric repeat amplification protocol (TRAP reaction)

According to the manufacturers' instructions, 2µl of cell extract was subjected to PCR reaction (negative controls were prepared using 2µl of lysis buffer only). Each reaction mix consisted of 25µl of reaction mixture (provided in the kit); 2µl of cell extract, and sterile deionised water (Sigma-Aldrich, UK) which was added to make up a final volume of 50µl. The conditions for PCR amplification were according to the manufacturers' instructions, using a protocol of: primer elongation at 25^0C for 15 min, telomerase inactivation at 94^0C for 5 min, followed by 30 cycles of amplification consisting of denaturation at 94^0C for 30 s, annealing at 50^0C for 30 s and extension at 72^0C for 90 s.

ELISA

5µl of the PCR product was added to denaturation reagent containing sodium hydroxide (<0.5%, provided in the kit). This was incubated at room temperature (15-25^0C) for 10 min. Hybridization buffer containing a DIG labeled detection probe

(provided in the kit) was added to each sample mixture and mixed thoroughly by vortexing. Each sample was transferred into 96-well microplate modules pre-coated with streptavidin and post-coated with blocking reagent (provided in the kit). This was incubated at 37^0C on a shaker at 300 rpm for 2h. The hybridization mixture was then removed, and the microplate washed 3 times with 250µl of washing buffer (provided in the kit). Anti-digoxigenin peroxidase antibody (anti-DIG-POD) was added and the microplate incubated at room temperature for 30 min whilst shaking at 300 rpm. The solution was then removed, and the microplate washed 5 times with 250µl of washing buffer (provided in the kit). TMB substrate (tetramethyl benzidine) was added, and the microplate incubated at room temperature for 15 min, whilst shaking at 300 rpm. Stop reagent was then added, and the absorbance measured using an ELISA microplate reader at 450nm (with a reference wavelength of 690nm), within 30 min. Samples are regarded as positive for telomerase activity if the difference in absorbance between the sample absorbance and negative control absorbance was higher than 0.2.

2.5.5 Measurement of protein concentration using the Bradford assay

The total protein concentration of the cell lysates was determined by the Bradford assay. The assay is based on the ability of Coomassie brilliant blue to combine with proteins to give a dye-protein complex with an absorption maximum of 595nm (Bradford 1976). The 1x Bradford reagent (Sigma Aldrich, Dorset, UK) was pre-warmed to ambient temperature prior to use. Bovine Serum Albumin (BSA, Sigma Aldrich, Dorset, UK) was used as the standard to determine the linear range of the assay (a serial dilution of BSA was prepared in double distilled water at a range of 1mg/ml to 0.0625mg/ml). 100µl of each BSA dilution was mixed with 3ml of Bradford reagent by vortexing. 100µl of deionised water was also mixed with 3ml of Bradford

reagent and this served as the blank that was used to ‘zero’ the spectrophotometer. Each mixture was incubated at room temperature for at least 5 min (but no longer than 1h). Samples were then transferred into clean disposable cuvettes (Fisher Scientific, UK) and absorbance measured at 595nm using a Cary 50 Bio UV–Visible Spectrophotometer (Varian Australia Pty Ltd, Australia). Each reading was performed in triplicate. A standard curve was constructed by plotting mean absorbance against BSA concentration as seen in figure 13. Dilutions of cells lysates were then prepared in distilled water, 100μl of which was added to 3ml of Bradford reagent and tested as described above. The protein concentration of the cell lysate was determined by comparing absorbance values against the standard curve. If the absorbance of the samples fell outside the linear range of the calibration curve, the samples were again diluted to obtain values within the linear range. The protein concentration of the original sample was then determined by multiplying the calculated concentration by the dilution factor. Figure 13 shows a typical standard curve obtained.

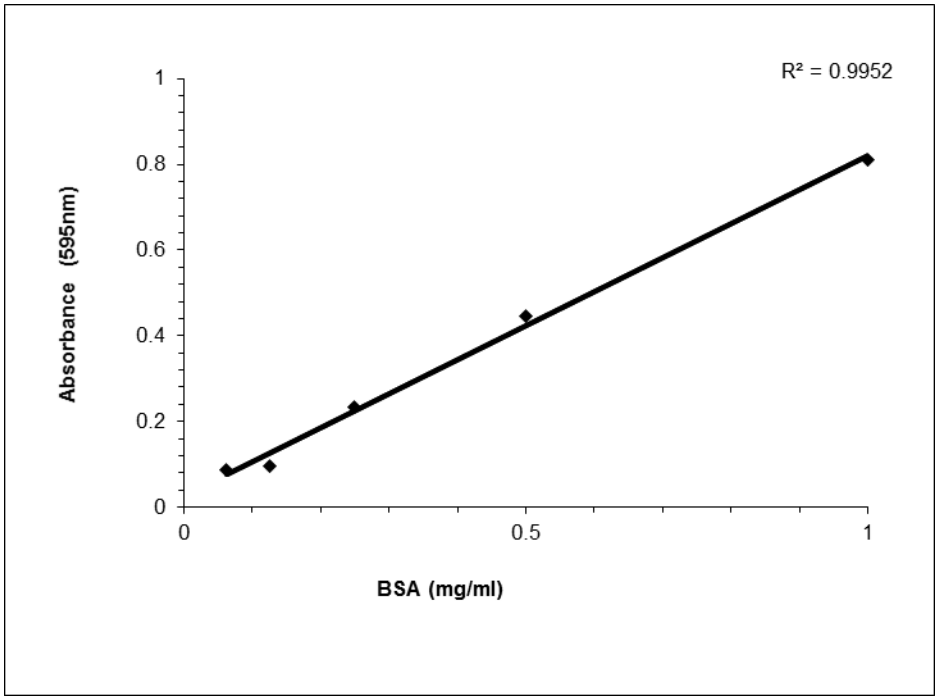


Figure 13 Representative example of a Bradford assay standard curve

2.5.6 Sensitivity of the TeloTAGGG Telomerase PCR ELISA kit

In order to determine the sensitivity of the kit, cell lysates produced from a cell line with known telomerase activity (A549) was serially diluted with lysis buffer and then assayed for telomerase activity. Varying concentrations of cells from a cell suspension were also added to saliva samples that had been collected from healthy volunteers as previously described. The samples were then centrifuged as described previously and the pellets were assessed for telomerase activity. The absorbance values obtained were plotted against the protein concentration of the cell lysate.

2.6 Methods specific to detection of telomerase activity

Ethics approval for study

Ethics approval was sought for the study. Approval was obtained from the Local Research and Ethics Committee (LREC) and the Research and Development Department of the Bradford Royal Infirmary. Approval was obtained for the collection and analysis of saliva samples and tumour tissue from patients diagnosed with head and neck squamous cell carcinoma and saliva samples from healthy controls (Appendix I). The period during which clinical data and samples collected spanned from 2005 – 2007.

Protocol for recruitment

Following ethics approval, patients newly diagnosed with HNSCC were identified in outpatients' clinic prior to surgery and recruited into the study. Information about the study was provided to the patients and their consent was sought prior to samples being collected (Appendix II – IV).

2.6.1 Collection of samples

Saliva samples were collected from recruited patients and healthy controls by asking individuals to gargle with either sodium lactate solution [Hartmann's Solution] or 0.9% sodium chloride solution (Baxter Healthcare Ltd, England). This was in the form of a mouth rinse. Samples were collected at least 2h after the patient ingested any food or drink. The solution was collected in a sterile tube, centrifuged at 1500*g* for 5 min and a pellet was obtained which was immediately frozen on dry ice. The pellet was stored at -80^{0}C until required (as described in section 1 using the A549 cell suspension). Formalin fixed, wax embedded specimens of the patient tissue

were obtained from the pathology department at Bradford Royal Infirmary. Histological stage was obtained from histology reports prepared by consultant pathologists in Bradford Teaching Hospitals NHS Trust.

2.6.2 Determination of best method of collection and storage of saliva samples

To determine the best method to use for collecting samples, healthy individuals were asked to gargle with either 3ml or 10ml of either 0.9% sodium chloride solution or Hartmann's solution. For the 3ml sample, a further 15ml of solution was added to the sample. Cells from a cell suspension of cell lines with known telomerase activity (freshly prepared $2x10^5$ cells from A549 cultures) were added to each sample, and centrifuged at 1500*g* for 5 min to obtain a pellet which was tested for telomerase activity

2.6.3 Determination of effect of saliva and collection fluid on the detection of telomerase activity

To ensure that the constituents of normal saliva did not affect the detection of telomerase activity (and also to mimic the saliva environment), healthy individuals with no past history of HNSCC were asked to gargle with either 0.9% sodium chloride solution or Hartmann's Solution. Cells from a cell suspension of a cell line with known telomerase activity ($2x10^5$ cells from A549 cultures) were added to the saliva samples, centrifuged and stored at -80^0C as described previously.

2.6.4 Preparation of saliva smears

Saliva samples were collected, and a cell pellet produced as described previously. The cells were smeared on clean slides using a disposable pipette. The slides were fixed immediately in 95% ethyl alcohol for 30 min or air-dried.

2.6.5 Haematoxylin and Eosin staining

In this widely used histological stain, the heamatoxylin component stains the cell nuclei blue black, and the eosin stains the cell cytoplasm red/pink. Haematoxylin and Eosin staining was performed as described below in table 6.

Step	Process	Time
1	Haematoxylin	10 minutes
2	Wash in running tap water	Briefly
3	Acid alcohol	5 seconds
4	Wash in running tap water	Briefly
5	Scott's tap water substuitute	1.5 minutes
6	1% aqueous eosin	1 minute
7	Wash in running tap water	Briefly
8	Drain slides	1 minute
9	Absolute ethanol	1 minute
10	Absolute ethanol	3 minutes
11	50%xylene ethanol	3 minutes
12	Xylene	3 minutes
13	Xylene	5 minutes
	Mount slide using DPX	

Table 6 Process of haematoxylin and eosin staining

2.6.6 Trypan blue viability

To determine cell viability in saliva samples, 0.4% Trypan blue stain was used. This method is based on the principle that live cells are able to exclude the dye and dead cells take up the dye. Approximately 100µl of saliva sample (it is an approximate volume due to the viscous nature of some saliva samples) mixed with same amount of trypan blue solution 0.4% (Sigma Aldrich, Dorset, UK) was placed in a Neubauer

haemocytometer chamber. This was then viewed using a low power objective (x10) to determine the uptake of tryphan blue, indicating viability of the cells.

Immunohistochemistry/immunocytochemistry

Immunocytochemistry and immunohistochemistry are techniques used to detect cellular antigens (immunocytochemistry) or tissues sections (immunohistochemistry). This is achieved by the use of specific antigen/antibody interactions that culminate in the attachment of a marker to the antigen. They allow reproducible and consistent demonstration of specific antigens with the minimum of background staining whilst preserving the integrity of the cellular/tissue architecture. The antigen/antibody complexes can then be visualised via light microscopy. hTERT protein is heterogeneously expressed in nucleoli of some normal and most cancer tissues and immunocytochemical and immunohistochemical staining was used to detect hTERT protein expression in cultured cells and tissue sections as described below.

2.6.7 Immunocytochemistry

Cell culture on microscopic slides

H157, an oral squamous cell carcinoma cell line shown to be telomerase positive and HUVEC cells, shown to be telomerase negative were used for immunocytochemistry. A cell count was performed and 500µl of the cell suspension at a density of 1 x 10^4 cells/ml (5000 cells) in medium DMEM for H157 cells and M199-M2154 medium for HUVEC cells was seeded into each well of a sterile eight - well chamber microscopic slide (Lab-Tek II chamber slide system, Fisher Scientific UK Ltd). The cells were incubated at 37C in a CO_2 enriched atmosphere of 5% CO_2. The media was changed after 24h in order to allow the cells to attach. After the cells

attained 60% confluence, the media was aspirated from the chamber slides and the cells were now ready for fixing as described below.

Immunocytochemistry on cultured cells

All procedures were carried out at room temperature, unless stated otherwise. Cells were cultured on multi – well microscopic slides as previously described.

Preparation of blocking sera

In order to make 10% goat serum, 1ml of freshly thawed goat serum (Invitrogen, Gibco, NY) was added to 9ml of PBS solution. The mixture was vortexed and then transferred to 1.5ml Eppendorf tubes. The Eppendorf tubes were then placed in a microcentrifuge and centrifuged at 1300g for 5 min to remove any particulate material. The resulting serum solution was stored at 4C and used for the blocking step. To make the 1% goat serum, 100µl of goat serum was added to 9.9ml of PBS solution. This was then vortexed and used to dilute the primary antibodies to the required concentration.

Preparation of the primary antibodies

The primary antibody (hTERT rabbit monoclonal antibody, Epitomics, CA, USA) was made up into the required dilutions (1:50, 1:100 and 1:200), using 1% goat serum. The diluted primary antibody was centrifuged for 5 min at 1300*g* to remove particulates and stored at 4^0C until needed.

Cell fixation

The culture medium was gently aspirated from each well and the cells were placed in ice-cold methanol at -20^0C for 10 min. The cells were then rehydrated in PBS for 2 min.

Blocking

A blocking solution consisting of 10% goat serum was prepared in PBS as previously described. 200µl of the blocking solution was added to each well. The slides were then incubated in a covered square dish for 1h at room temperature.

Staining

After 1 h incubation in the blocking solution, each well was drained carefully of the goat serum and 100µl of the appropriately diluted primary antibody (1:50, 1:100 and 1:200) was added to 2 wells. 100µl of goat serum only was added a further 2 wells to serve as the negative control. The slides were incubated with the primary antibody overnight at 4^0C. The next day, the cells were washed with 200µl of PBS for 5 min and this was repeated twice. The DakoCytomation LSAB 2 System-HRP® kit (DakoCytomation, Glostrup, Denmark) was used for staining. Secondary antibody (100µl obtained from the kit), was added to each chamber and incubated for 15 min at room temperature. The secondary antibody was aspirated, and the cells were then washed with 200µl PBS for 5 min and this was repeated twice. Streptavidin - peroxidise solution (100µl from kit) was added to each well and incubated for a further 20 min at room temperature. The streptavidin - peroxidise solution was aspirated, and the cells were then washed with 200µl of PBS for 5 min and this was repeated twice. 200µl of distilled water was added to each well and incubated for 2 min. The distilled water was aspirated and 200µl of DAB substrate - chromogen solution (provided in the kit) was added to each well and incubated for 10 min. Each chamber was then washed 3 times with 200µl of distilled water. The chambers were then carefully removed from the slide and the cells were mounted.

Mounting the microscopic slides

The cells were mounted using Glycergel® mounting medium (DakoCytomation, Glostrup, Denmark). The glycergel was warmed in a 40^0C water bath to liquefy and a small amount was then placed on the centre of a 22 x 50mm coverslip (VWR International, Poole, UK) and carefully placed on the wet slide. The slide was then left to set for 90 min.

2.6.8 Immunohistochemistry

A549 xenografts were used for immunohistochemistry as A549 cells have been shown to have telomerase activity (see figure 14 below). A549 xenografts and formalin fixed/wax embedded specimens of patient tissue samples were used. The A549 xenografts were derived from subcutaneously grown tumours in NCR/Nu mice that had been surgically excised from mice under anaesthesia. All procedures were carried out by ICT staff and conducted under United Kingdom Co-ordinating Committee on Cancer Research (UKCCR) guidelines (1998) and Home Office project licence (40/2585). The formalin fixed/wax embedded specimens of the patient tissue samples were obtained from the pathology department at Bradford Royal Infirmary. Tissue samples were not available for all patients in the study.

Preparation of A549 xenograft

The freshly excised A549 xenograft tumour samples were immersed in 10% formalin in 0.01M phosphate buffered saline for 24 h in order to achieve tissue fixation. The samples were then loaded onto histology biopsy cassettes and processed in an automatic tissue processor using the following processing cycles: 70% ethanol for 60 min, 80% ethanol for 60 min, 95% ethanol for 60 min, 100 % ethanol for 60 min, fresh 100% ethanol for 60 min, fresh 100% ethanol for 120 min, 100% xylene for 60

min, fresh 100% xylene for 60 min, fresh 100% xylene for 120 min, paraffin wax for 150 min and then fresh paraffin wax for a further 150 min. The fixed tissue samples were then embedded in paraffin blocks.

Preparation of tissue sections

Formalin fixed paraffin embedded tissue blocks of A549 xenografts and that of patients were cooled for a minimum of 20 min in the -20^{0}C freezer. The blocks were then cut using a microtome into 5 micron sections. Tissue sections were mounted onto BDH 1.2 mm twin frosted slides pre-coated with 3-amino-propyl-triethoxy-silane (APES) via a warm water bath at 48^{0}C and left on a heating plate overnight to dry.

Deparaffinization and Rehydration

In order to soften the wax, the slides were placed in the oven at 65^{0}C for 1h. The slides were then deparaffinised and hydrated as described below in table 7.

Step	Process	Time
1	Xylene x2	5 min
2	Absolute ethanol x2	5 min
3	95% ethanol	5 min
4	70% ethanol	5 min
5	50 % ethanol	5 min
6	30% ethanol	5 min
7	water	5 min on shaker
8	TBST	5 min on shaker

Table 7 Process of deparaffinisation and hydration of slides

Antigen retrieval

Antigen retrieval solution (citric acid buffer, pH 6.0) was prepared using citric acid and double distilled water (10mM citric acid + 0.05% Tween + double distilled water). Two methods of antigen retrieval were investigated. The first method was using the microwave and the second method was using the oven. Different concentrations of primary antibody were also used in both methods (1:200, 1:100 and 1:50). 1:50 dilution was the primary antibody concentration selected for the patient tissue samples as preliminary studies demonstrated that this gave the best degree of staining.

Microwave method of antigen retrieval

In order to determine the effect of microwaving on the antigen retrieval process, different methods of antigen retrieval using the microwave was performed: a) 20 min microwave, b) 10 min microwave, c) no microwave. The slides were immersed in a dish containing antigen retrieval solution and heated in the microwave for 10 or 20 min. Two slides were immersed in the antigen retrieval solution but were not placed in the microwave (no microwave). The slides were then left in the antigen retrieval solution to cool for 30 min.

Oven method of antigen retrieval

Antigen retrieval solution was warmed up to 90^0C in the oven and the slides were immersed in the antigen retrieval solution for 40 min. The slides were then cooled quickly in running water for 10 min.

Staining

Slides were washed with TBST (Tris-buffered saline + Tween 20, Sigma-Aldrich Company Ltd, Poole, Dorset, UK) for 10 min. Endogenase peroxidase was inactivated by adding 1% (v/v) hydrogen peroxide to each section for 30 min. Slides were then washed 3 times with TBST (for 3 mins per wash). Excess TBST was carefully blotted off with tissue avoiding touching the sections, and the sections were then encircled using a PAP pen (DAKO Ltd, Cambridgeshire, UK) in order to create a hydrophobic wall around the sections. The 1% and 10% goat serum were prepared as previously described. 200µl of 10% goat serum was used as blocking solution, added to each section, and this was incubated for 1h. The primary antibody (hTERT rabbit monoclonal antibody, Epitomics, CA, USA) was prepared by making up 1:50, 1:100 and 1:200 dilutions with 1% goat serum (as described previously). After 1h incubation with 10% goat serum, each slide was drained carefully of the goat serum and 100µl of primary antibody for each dilution was added to a section on the slides. 100µl of goat serum was also added to a section as the negative control. The slides were incubated with the primary antibody overnight at 40^0C.

The next day, the slides were washed in distilled water, and then washed for 3 min each, 3 times with TBST. Secondary antibody (Biotinylated Anti-Rabbit IgG, made in goat, Vector Laboratories LTD, Peterborough, UK) was prepared with 1% goat serum making up a 1:200 dilution. This was applied to each section and incubated at room temperature for 1h. The secondary antibody was then removed, and the slides washed for 3 min, 3 times in TBST. The Vectastain® ABC reagent (Vectastain Avidin: Biotiylated enzyme complex, ABC kit, Vector Laboratories LTD, Peterborough, UK) was freshly prepared according to manufacturer's instructions by adding 1 drop of solution A and 1 drop of solution B to 5mls of PBS. This was left to

stand for 30 min. The ABC reagent was then added to each section and incubated for 30 min at room temperature. The ABC reagent was removed, and the slides washed in distilled water. The slides were then washed for 3 min, 3 times in TBST. DAB peroxidase substrate (DAB peroxidase substrate kit, Vector Laboratories LTD, Peterborough, UK) was freshly prepared according to manufacturer's instructions, by adding 1 drop of buffer stock, 2 drops of DAB stock solution and 2 drops of H_2O_2 stock solution into 2.5ml distilled H_2O. This was then added to each section for 5 min at room temperature until colour development. Each slide was rinsed with distilled water and then rinsed with tap water for 5 min.

Counterstaining

The sections were counterstained with Harris haematoxylin. The slides were placed in haematoxylin for 20 s and immediately washed in running tap water for 1min. The sections were then placed in Scott's tap water for 1 min. The slides were dehydrated as described below in table 8.

Step	Process	Time
1	70% ethanol	3 min
2	90% ethanol	3 min
3	Absolute ethanol	3 min
4	Absolute ethanol	3 min
5	50% xylene/ethanol	3 min
6	Xylene	3 min
7	Xylene	5 min

Table 8 Process of dehydration of slides

Mounting of slides

The slides were then mounted using DPX® mounting medium (VWR International, Poole, UK). After dehydration, the slides were removed from the xylene one by one, the edges were dried, a small drop of DPX was placed on a 22 x 50mm coverslip and this was carefully applied to the slide pressing gently to remove air bubbles. The slides were left flat to set for 1h. The staining was observed under a light microscope.

2.7 Methods specific to the inhibition of telomerase study

2.7.1 Methodology for telomerase inhibition by compounds

Berberine chloride has been shown to be a moderate inhibitor of telomerase (Naasani et al 1999). On this basis it was selected as a positive control to develop an experimental technique for screening novel compounds. In order to assess the ability of berberine chloride to inhibit telomerase, an initial stock concentration of 1mM was prepared with water. This was added to the cell lysates (freshly produced from $2x10^5$ cells), to make final concentrations of 200, 20, 2 and 0.2µM of berberine chloride, and each lysate was then incubated at 37^0C for 2h. The 2h time period was chosen based on the results of the stability of the telomerase enzyme at 37 ^{0}C with time (section 4.4.1). The absorbance values obtained were plotted against the berberine chloride concentrations. The control was cell lysate with an equal volume of water as drug volume, incubated at 37^0C for 2h. After incubation, 2µl of drug/lysate mixture was added to the PCR reaction mixture and subjected to PCR/ELISA as described in section 3.1.4. Other established cytotoxic drugs, (doxorubicin, 5 fluorouracil, and Cisplatin) were also assessed with the above methodology. The novel compounds were then screened using the above methodology.

2.7.2 Evaluation of selected compounds as non-specific inhibitors of telomerase

In order to ensure that the inhibition detected was a result of specific telomerase inhibition rather than a false positive/nonspecific inhibition of Taq polymerase, each compound was subjected to PCR reaction/agarose gel electrophoresis, to detect the presence or absence of β actin. The absence of β actin suggested the compound inhibited Taq polymerase (and as such, the PCR reaction). Detailed methodology is described below.

RNA extraction

Using A549 cells, a cell pellet was prepared as described in section 3.1.4. Using the RNA extraction kit (RNAqueous®-Micro, Ambion – Applied Biosystems, Warrington, UK), the cells were lysed by adding 100µl of lysis solution. 50µl of 100% ethanol was added to the lysate and vortexed briefly. The lysate/ethanol mixture was then loaded onto a MicroFiltre Cartridge Assembly (consisting of filter and collection tube, from kit) and with the cap on, was centrifuged at 4^0C for 1 min at 16000*g*. This allows the entire sample to pass through the filter into the collection tube and the solution from the tube discarded. The RNA binds to the filter in the microfilter cartridge. The filter was washed 3 times with 180µl of wash solution (working solution mixed with ethanol, from kit) by adding the wash solution to the filter and centrifuged as above. The contents of the collection tube were discarded each time. The microfiltre was transferred into a microelution tube (from kit).10µl of elution solution (provided with kit and preheated to 75^0C), was added to the filter, this was left to sit for 1 minute at room temperature, then centrifuged at 4^0C for 1 min at 16000*g* to elute the RNA from the filter. DNA contamination was removed from the eluate by adding 2µl of 10X DNase buffer and 1µl of DNase I (provided with kit). This was placed in a water bath for 30 min at 37^0C. The DNase was inactivated by heating the tube at 75^0C for 15 min.

RNA analysis

The quantity and yield of RNA extraction was measured using a Cary 50 Bio UV–Visible spectrophotometer (Varian Australia Pty Ltd, Australia). This was determined by diluting 2µl of RNA in 198µl of distilled water. The distilled water alone was used as the blank sample. The RNA sample absorbance was measured at the ultraviolet wavelengths of 260nm (A_{260}) and 280nm (A_{280}). An A_{260} of 1 is equivalent to 40 µg/ml

and the RNA quantity (µg/ml) was calculated by using the absorbance value at 260nm, using the following formula

[$Absorbance_{260}$ X 40 X dilution factor (of RNA)].

The ratio of the sample absorbance at 260nm (absorbance for RNA) and 280nm (absorbance for proteins) was calculated and ratios of 1.7 - 2.1 are essentially free of protein contamination.

cDNA synthesis (reverse transcription)

Reverse transcription of the RNA was performed using the RETROscript® Kit (RNAqueous®-Micro, Ambion – Applied Biosystems, Warrington, UK). The RNA was first of all denatured with heat by adding 2µl of RNA to 2µl of oligo (dT) and 8µl of nuclease-free water to make a total volume of 12µl (2 tubes of above solution were prepared). Each tube was incubated at 75^0C for 3 min, and then cooled on ice. A reaction mixture containing 2µl of 10X buffer, 4µl of dNTP mix (premixed aqueous solution of dATP, dCTP, dGTP, dTTP at a final concentration of 10mM), 1µl of RNase inhibitor, 1µl of MMLV- RT and 12µl of RNA/oligo(dT) mixture made up to 20µl with nuclease free water and labelled as tube 1. Tube 2 contained the above without MMLV-RT. This served as a control to detect DNA contamination in the reaction. Each tube was incubated at 43^0C for 1h. The tubes were then incubated at 92^0C for 10 min in order to inactivate the reverse transcriptase. The cDNA product was stored at -20^0C until needed.

Amplification of β actin cDNA by Polymerase chain reaction (PCR)

The PCR kit used was Taq PCR Core Kit (Qiagen, Crawley, UK). β actin primers were supplied by MWG Biotech, Ebersberg, Germany. The specificity of primers was

checked via a BLAST search (NCBI website) and was shown to be specific to β actin. The primers used were as follows:

Forward 5' – CCA-CGA-AAC-TAC-CTT-CAA-CTC-C – 3'

Reverse 5' – TCA-TAC-TCC-TGC-TTG-CTG-ATC-C – 3'

Each primer was made up to 10μM stock in distilled water and added to the PCR reaction mix to give a final concentration of 0.2μM. The primer stock solution was stored at -20^{0}C.

To a sterile PCR tube (AXYGEN Scientific, CA) on ice, 1μl of dNTP stock solution, 1μl of each primer (10μM of reverse and forward primers), 5μl of Taq PCR 10X buffer, 0.25μl of Taq DNA Polymerase, 1μl of cDNA and sterile double distilled water were added to make up a 50μl reaction mixture. First, a reaction mix (without cDNA) was prepared to avoid tube to tube inconsistency (master mix). For the reaction mix containing the novel compounds, each compound was made up to 1mM stock (using either DMSO or water) and added to the PCR mix to give a final concentration of 200μM. The PCR conditions used were: initial denaturation at 94 ^{0}C for 1 min, denaturation at 94^{0}C for 30s, annealing at 60^{0}C for 45s, elongation at 72^{0}C for 30s, final elongation at 72^{0}C for 5 min and cooling at 15^{0}C. The reaction was carried out for 35 cycles.

PCR controls used included: 2 negative controls – one without RT (reverse transcriptase) and the other without cDNA; and a positive control with added cDNA.

Each 1μl of each PCR product was added to 2μl of loading dye (Sigma-Aldrich, Dorset, UK) and run in parallel with a 1kb plus DNA ladder (Invitrogen, UK) on a 2% agarose gel (agarose in 0.5X Tris-Borate-EDTA-buffer) stained with 0.5 μg/ml

ethidium bromide (sigma-Aldrich, Dorset, UK) at 100V for 1 hour. The gel was then scanned using Multimager Fluor-S (Bio-Rad, Hertfordshire, UK).

2.7.3 The chemosensitivity assay (MTT)

The MTT assay was used to determine cell survival. This assay relies upon measuring the cellular reduction of a yellow, water soluble tetrazolium salt, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) via succinate-dependent conversion by mitochondrial enzymes to a purple, water insoluble formazan product that can be detected at visible wave length on a multiwell scanning spectrophotometer following dissolution in an organic solvent (Mosmann 1983).

Validity of the MTT assay

The validity of the MTT assay was assessed by examining the linear relationship between cell number and absorbance values. Serial cell dilutions (in a total volume of 200µl of media), containing different concentrations of cells were seeded into 96-well plates (8 wells per dilution). One lane (with 8 wells) was prepared with just media (200µl/well) and no cells as a blank. This was followed by the addition of 20µl volume of MTT (5mg/ml) to each well. The plates were incubated in the dark for 4h at 37^0C. The supernatant was then carefully removed and discarded leaving the reduced formazan crystals at the bottom of the wells. 150µl of DMSO was added to each well to dissolve the crystals. The absorbance was read immediately at 540nm using a Labsystems Multiscan Plus Spectrophotometer (Labsystems group, UK). The mean absorbance value was calculated for each dilution and the blank value was subtracted from each calculated mean absorbance. The adjusted mean absorbance was then plotted against the cell numbers. A linear regression was calculated to check the linear relationship between absorbance and cell number.

Chemosensitivity measurements

1000 cells per well were seeded into 96-well plates, preparing lanes 2 to 12. Lane 1 was left as a blank (with media and no cells). For the HUVEC cells, 20000 cells ($2x10^4$ cells) per well were seeded into 96-well plates. The cells were then incubated at 37^0C overnight to attach. The media was carefully removed and replaced with fresh media containing a range of concentrations of selected compounds serially diluted (except lane 1 which was left with media alone and lane 2 (control) with cells in media but no compound). The cells in lanes 3 to 12 were exposed to compounds for 2h and 96h. After 2h incubation at 37^0C, the cells were washed twice with HBSS, fresh media was added, and the cells incubated for a further 96h at 37^0C. The cells exposed to compounds for 96h were left undisturbed. Following incubation, cell survival was determined by using the MTT assay as described previously. The mean value was calculated for 8 absorbance measurements per drug dilution lane (and control lane) and the mean of the blank measurements subtracted from each mean absorbance value. The adjusted absorbance values were used to calculate the percentage cell survival using the control values as 100% cell survival as follows:

% survival = (Absorbance of treated cells/Absorbance of control cells) x 100

This % survival was plotted against the drug concentration range and IC_{50} (concentration required to kill 50% of cells) was determined.

2.7.4 Evaluation of the mechanism of telomerase inhibition by the selected compounds

As described previously, chromosomal ends are protected by the telomere which forms a cap at the end of the chromosomes. As well as the effect on the elongation

of the telomere ends, telomerase also stabilizes the telomere through its additional stabilization of the telomere cap (Blasco 2002, Masutomi et al 2003, Sung et al 2005). The differences in telomerase activity and telomere dynamics between cancer and normal cells and also the telomere/telomerase complex offers multiple possibilities for inhibition. Further evaluation of the mechanism of action of the selected compounds on the telomere/telomerase activity was investigated by looking at the ability of compounds to induce cellular senescence using the senescence assay. Cells lines selected for this study were the PC3 and A549 cell lines.

2.7.5 Senescence associated β galactosidase staining

Senesecence is an arrested state in which the cell remains viable but display altered patterns of gene and protein expression. Interference with telomerase architecture has been shown to evoke cell cycle arrest and senescence (Leonetti et al, 2004).

Senescence assay

The senescence assay was used to determine if the selected compounds which showed telomerase inhibition exerted growth inhibitory effects on cancer cells. A commonly used marker for senescence *in vitro* is the appearance of senescence associated β – galactosidase activity. Senescence was assayed using the senescence β – galactosidase staining kit (New England Biolabs (UK) Ltd, Cell signalling technology), based on a procedure first described by Dimri et al in 1995. The assay relies on the ability of senescent cells to express β galactosidase and this can be detected in single cells by X gal which forms a local blue precipitate upon cleavage. According to the manufacturer's instructions, cells were seeded into 6-well plates and incubated at 37^0C overnight to attach. The growth medium was then carefully removed from the cells and each well washed with 2ml of 1X PBS. The

cells were then fixed with 1ml of 1X fixative solution for 15 min at room temperature. The staining solution was prepared using 20mg/ml of X-gal in DMF, staining solution, staining supplement A and B (all provided in the kit). After 15 min, the cells were washed twice with 2ml of 1X PBS and then 1 ml of staining solution was added to each well and incubated overnight at 37^0C. Development of blue colour was assessed under a light microscope. The plates were overlaid with 70% glycerol and stored at 4^0C.

Plating of cells

In order to determine the best concentration of cells to use for the senescence assay, 10000 cells, 1000 cells and 100 cells were seeded into 6 well plates and incubated for 5, 10 and 15 days each. On day 5, the plate was read for the 5 day incubation and the media was changed for the 10 day incubation. On day 10, the plate was read for the 10 day incubation and the media was changed for the15 day incubation. On day 15, the plate was read for 15 day incubation.1000 cells per well was the cell number selected for plating as preliminary studies demonstrated that this gave the best cell confluence (data not shown).

Determination of minimally toxic drug concentrations

Chemosensitivity measurements using the MTT assay were made to determine sub-toxic doses (IC 10 - doses at which 90% of the cells are viable) of the compounds. 10 fold dilutions of the sub-toxic doses of the compounds were prepared and then added to the cells in order to demonstrate the senescence effect.

Methodology for β galactosidase staining following drug exposure.

In vitro studies have shown that arsenic induces DNA and chromosome damage (Zhang et al, 1999; Chou et al 2001). On this basis, it was selected as a control to exhibit senescence. 1000 cells per well were seeded into 6-well plates and incubated at 37^0C overnight to attach. The media was carefully removed and replaced with fresh media containing 10 fold dilutions of the sub-toxic doses of arsenic and the compounds (well 1 was left with media alone to serve as a control). The media was replaced every five days with fresh media containing the same concentrations of compounds (except well 1 which was replaced with media alone). The cells were exposed to arsenic and the compounds for 5, 10 and 15 days at 37^0C, after which β galactosidase expression was assessed as described above.

3 RESULTS

Telomerase assay

3.1 Detection of telomerase activity

Telomerase activity was initially determined using a known telomerase positive A549 cell line. A549 cells were counted and lysates were produced from 2 x 10^5 cells which were then assayed for telomerase activity using the telomerase PCR ELISA kit. Telomerase activity is represented by the absorbance value obtained in this assay. Figure 14 shows a representative result. The A549 cell line gave high absorbance values indicating the presence of telomerase activity, comparable to the positive control provided in the kit. In many instances there were day to day variations in the absolute absorbance values obtained in each experiment (depending on the age of the kit) and therefore the results shown in figure 14 below and in other figures are from individual experiments.

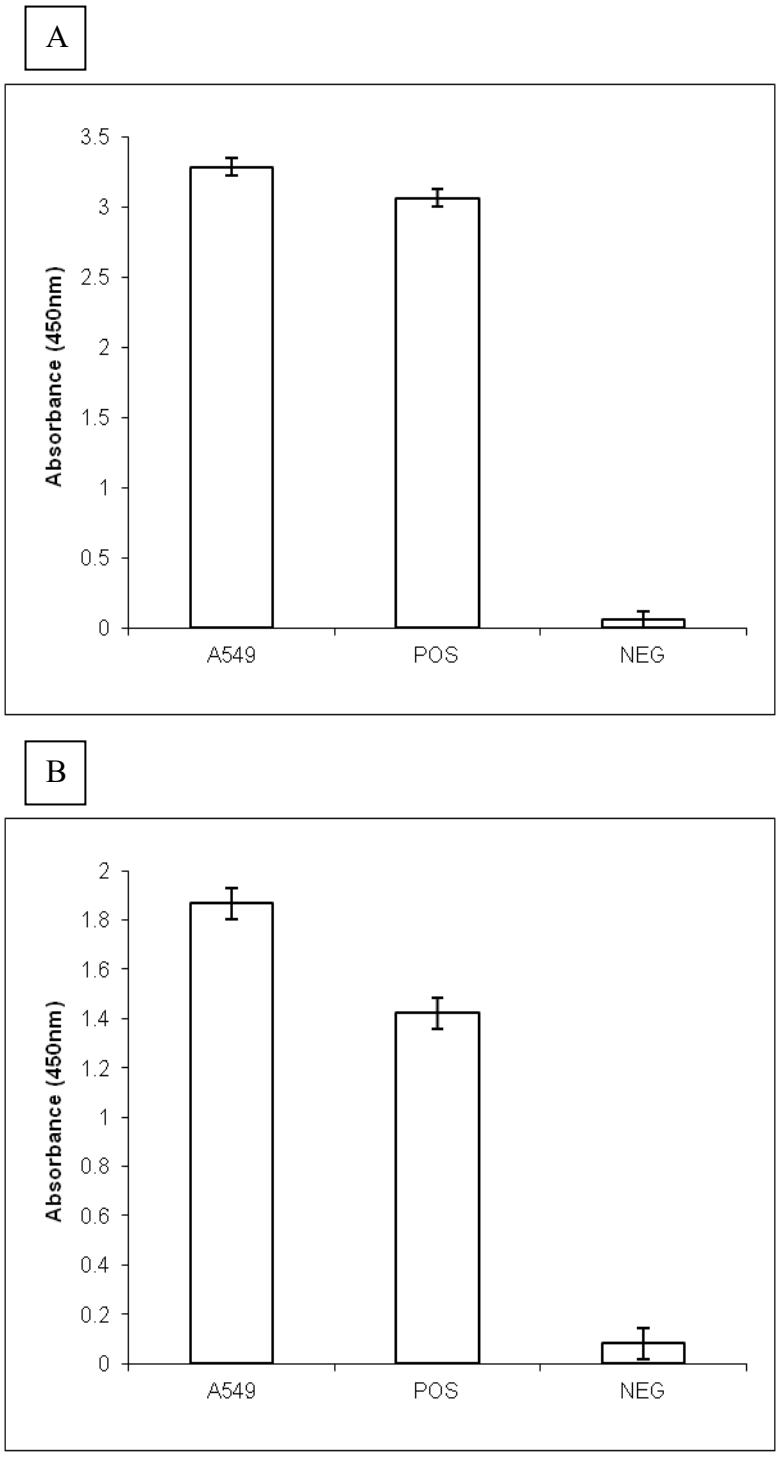


Figure 14 A, B Telomerase activity in A549 cells. Results show absorbance values from duplicate determinations in single experiments. There were variations in absolute absorbance values obtained in each experiment as this depended on the age of the kit. POS represents positive control (cell extract) and NEG represents negative control (lysis buffer) as supplied by the manufacturers of the kit.

3.2 Sensitivity of telomerase PCR ELISA kit

To investigate the quantitative nature and the sensitivity of the telomerase assay using the telomerase PCR ELISA kit, telomerase activity was measured in:

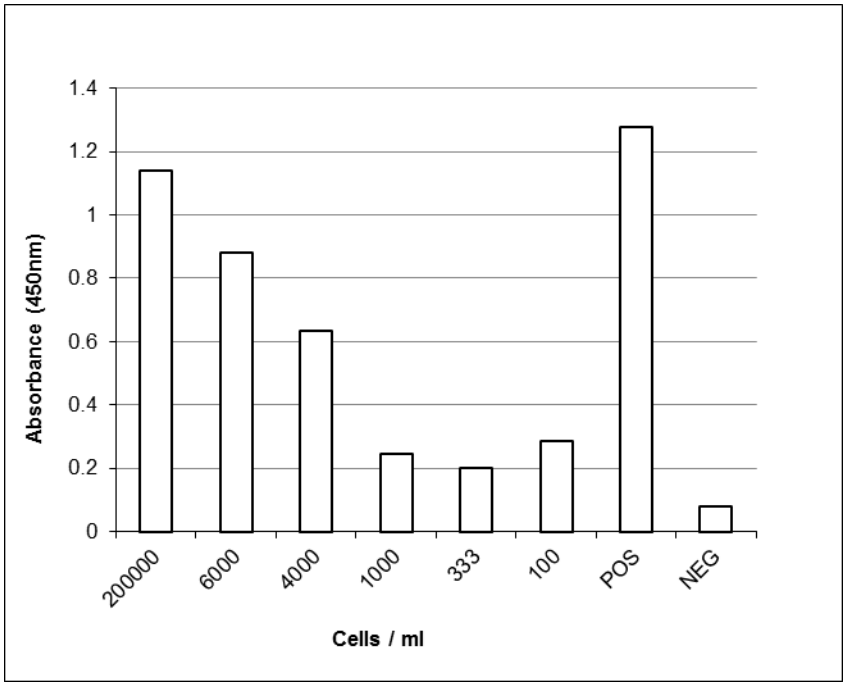
1. A549 cell lysates produced from serial dilutions of a known number of cells (Figure 15).

2. A549 cell lysates produced from an initial suspension with a known number of cells which were then serially diluted with lysis buffer (figure 16). The relationship between the protein content of the serially diluted cell lysates and the absorbance obtained in the telomerase assay of these lysates was also investigated (Figure 17).

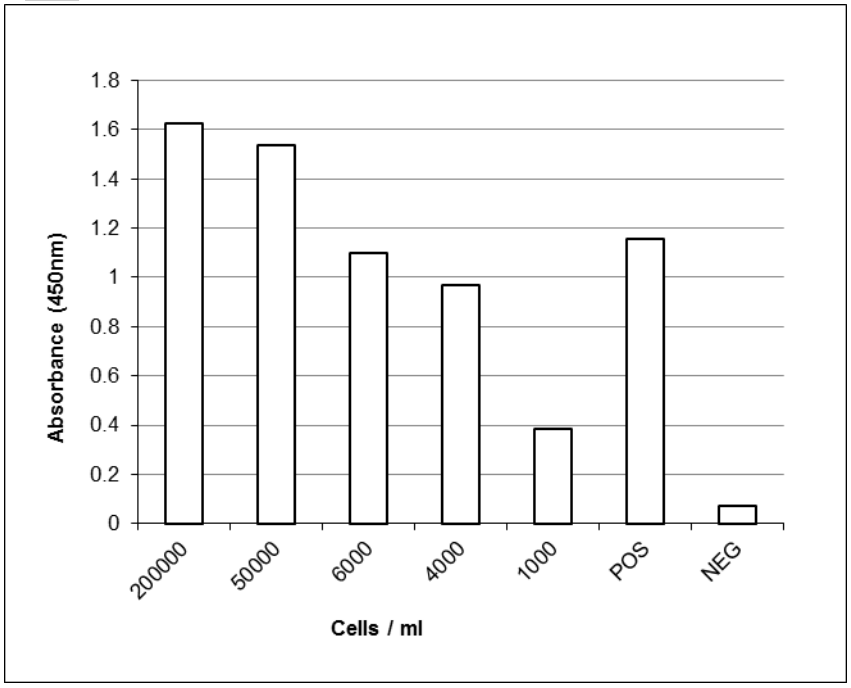
Serial dilution of cells

A549 cells were counted and 2 x 10^5 cells in suspension were serially diluted with media to give varying cell numbers per ml. Each ml of cell suspension was then centrifuged, and the cell pellets were lysed. 2µl volume of each lysate (see section 3.1.4) was assayed for telomerase activity in 3 separate experiments using lysates from different cell counts for each experiment. The results show a reduction in absorbance values as the cell counts reduced (Figure 15 A, B, C), however, when the cell counts fell below 1000 cells/ml, there was a sharp drop and inconsistency in the absorbance values detected

A


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance (450nm)
200000
6000
4000
1000
333
100
POS
NEG
Cells / ml

B


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance (450nm)
200000
50000
6000
4000
1000
POS
NEG
Cells / ml

C

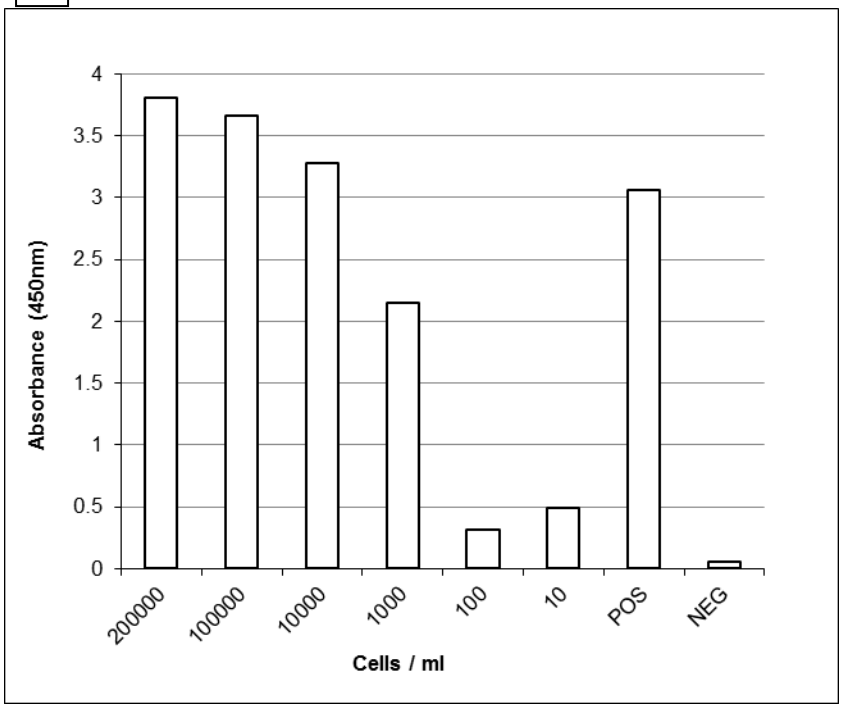


Figure 15 A, B & C are three independent experiments showing the relationship between cell concentration (used to prepare the lysate) and absorbance in the telomerase assay. 2µl of each lysate was used in the PCR reaction. Results show absorbance values from single determinations in each experiment.

Serial dilution of cell lysate

To investigate the effect of serial dilution of the cell lysate (and protein concentration), a cell lysate produced from 2 x 10^5 A549 cells, of known protein concentration (0.8µg/µl), was diluted with lysis buffer. 10µl volumes of lysate was serially diluted with 10µl of lysis buffer (with corresponding dilution of the protein concentration). 2µl of the diluted lysates was then used for the PCR reaction and assayed for telomerase activity. This provided a series of dilutions ranging from 1:2 to 1:10000 as shown in figures 16 and 17 (1:2 corresponded to 200000 cells). 200000 cells corresponded to a protein concentration of 0.8µg/µl. The results show a reduction in absorbance values as the cell lysate was diluted (Figure 16). A concentration of 0.02µg/µl of cell lysate equates to 5000 cells. Figure 17 shows the corresponding results using protein concentration of the lysates in place of serial cell dilution on the x axis. Detectable telomerase activity was demonstrated with a

protein concentration of 0.02µg/µl of lysate (produced from 5000 cells), in the PCR reaction corresponding to total protein of 40ng in the reaction itself.

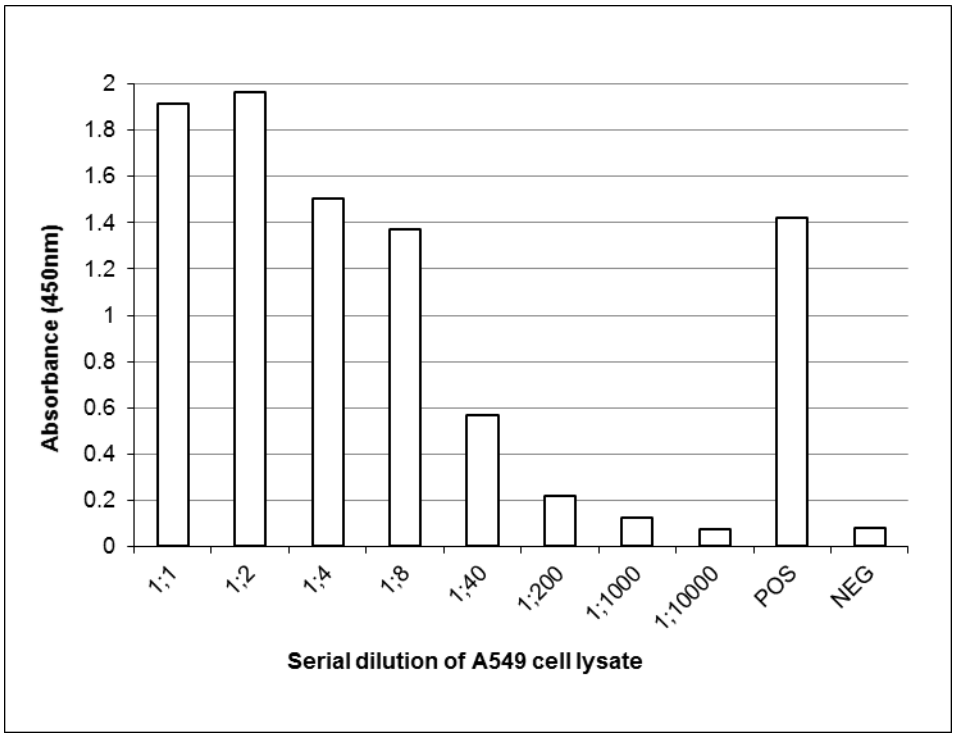


Figure 16 Relationship between serial dilution of A549 cell lysate / protein concentration and absorbance in the telomerase assay. 1:1 ratio represents lysate produced from 200000 cells which was serially diluted (1:1 = 200000 cells which has a protein concentration of 0.8 µg/µl).

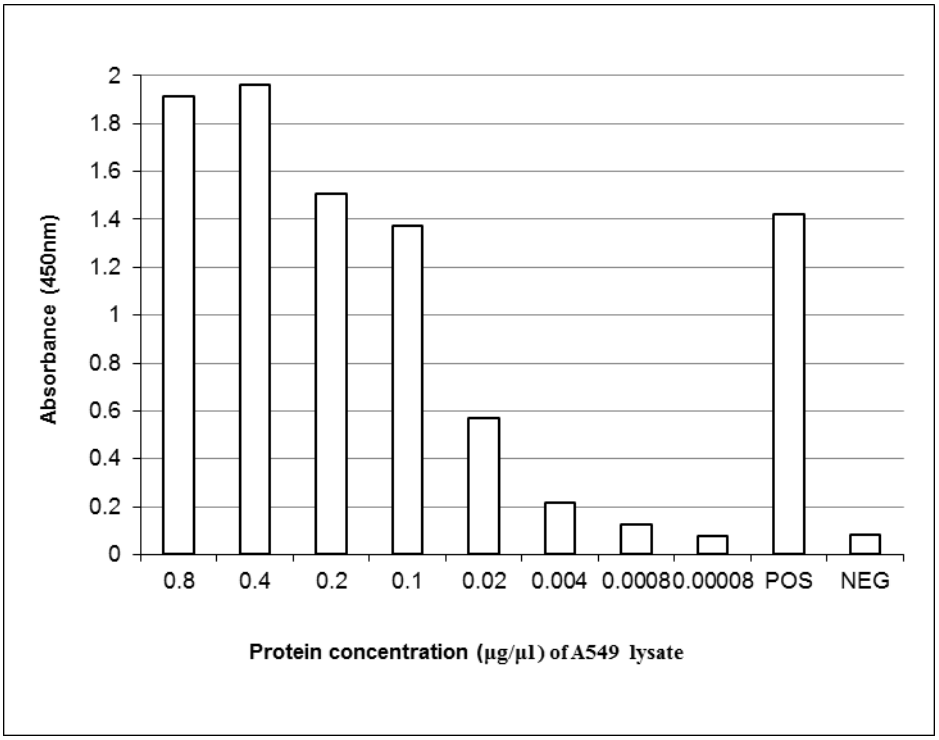


Figure 17 Protein concentration of the serially diluted lysates shown in figure 16. Results show absorbance values from single determinations in a single experiment.

3.3 Detection of telomerase activity in saliva

3.3.1 Epithelial cells in saliva

In order to assess the cell composition of saliva, a series of saliva smears obtained from 2 healthy volunteers were prepared and stained with haematoxylin and eosin. Figure 18 is a representative smear showing the presence of squamous epithelial cells and inflammatory cells.

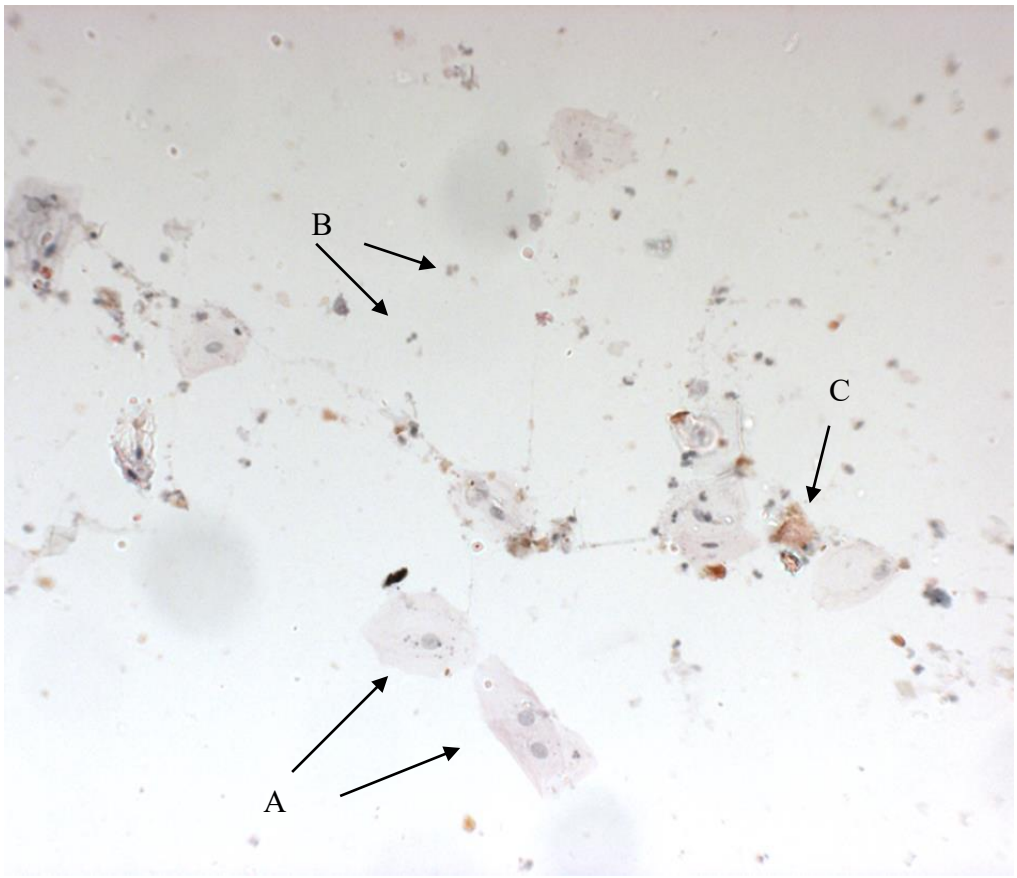


Figure 18 Haematoxylin and Eosin stain of saliva smear showing: A - nucleated squamous epithelial cells which appear flat with irregular shapes, B - inflammatory cells and C – debris.

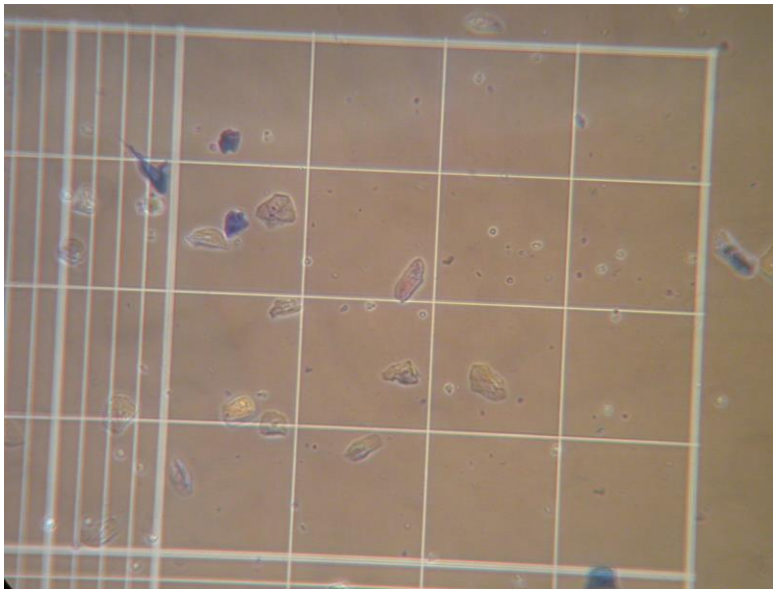


Figure 19 Trypan blue exclusion showing nonviable and viable cells from healthy volunteers

The viability of these cells was subsequently determined using trypan blue exclusion shown in figure 19 above. Of the 27 cells, only 2 cells (7% of all the cells) showed uptake of tryphan blue, indicating 93% viability of the cells. The results suggest majority of the cells in the oral rinses were viable.

3.3.2 Determination of telomerase activity in saliva

In the following experiments, validation of the telomerase assay was performed in human saliva samples 'spiked' with telomerase positive A549 cells.

Effect of saliva and solutions for collection of saliva on telomerase assay

In order to assess the effect saliva, and the effect the solutions used for the collection of saliva may have on the TRAP assay, telomerase activity in A549 cells was determined in the absence and presence of saliva samples collected from healthy volunteers. This was achieved by adding a known number of A549 cells ($2x10^5$) to oral rinses obtained from the healthy volunteers (by asking them to gargle with either Hartmann's or saline solutions). The A549 cells were those prepared as cells extracts and stored at -80^0C. The results of a single experiment are shown in

figure 20. There were no obvious differences between the absorbance obtained using lysates from: (i) A549 cells alone, (ii) A549 cells added to oral rinses obtained using either Hartmann's or saline solution or (iii) A549 cells added directly to Hartmann's or saline solution (without oral rinses). Neither saliva, Hartmann's nor saline solution had any effect on the telomerase assay. The absorbance obtained did not appear to be affected by the storage condition of the cell pellet at -80^{0}C.

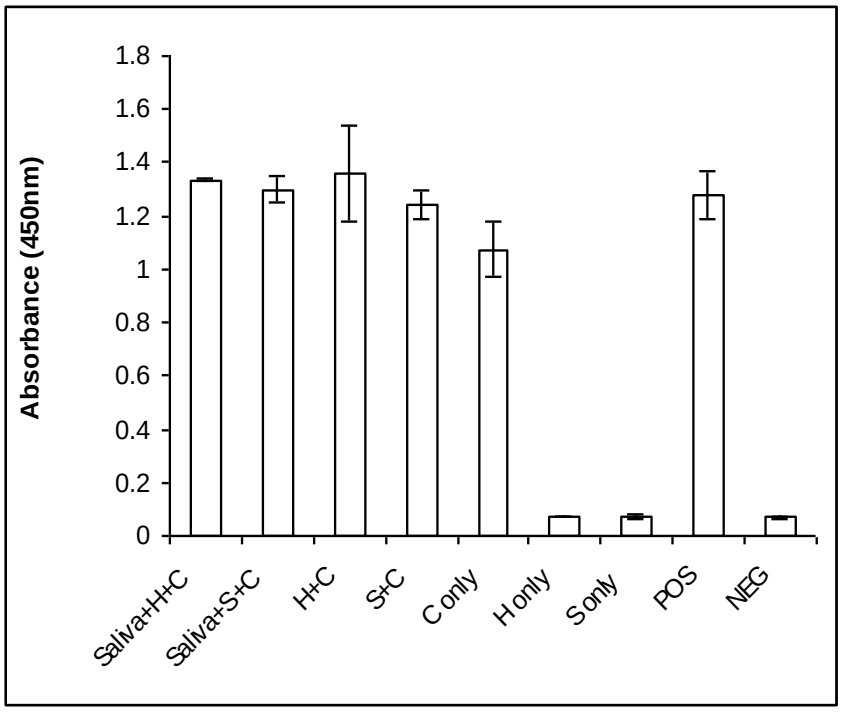


Figure 20 Effect of saliva and the solutions (Hartmann's and saline) used for saliva collection on the telomerase assay. C – A549 cells in absence of saliva; H – Hartmann's solution, S – saline solution; H+C/S+C – addition of cells to Hartmann's / saline solution in absence of saliva; saliva +H+C/saliva +S+C – addition of cells to saliva collected by gargling with Hartmann's solution/saline solution. The results are representative results showing the absorbance values from duplicate determinations in a single experiment.

Effect of the method of saliva collection on telomerase assay

In order to determine if the collection protocol affected the telomerase activity detected, oral rinses were collected from healthy volunteers by gargling with 3mls or 10mls of Hartmann's or saline solution. The results show similar absorbance values with each method of collection (Figure 21). Therefore, future studies were designed such that 10mls of saline was used as the solution to gargle.

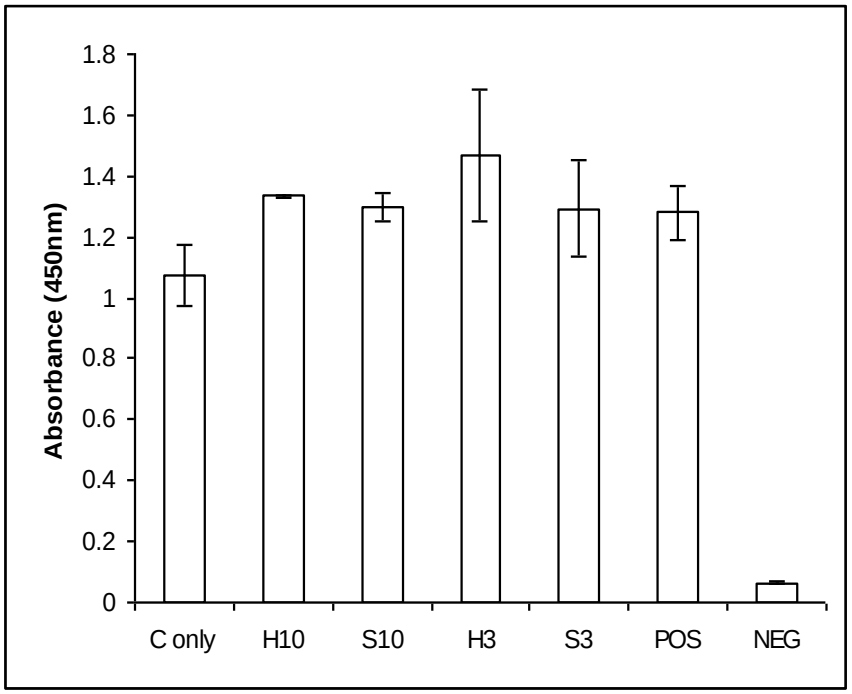


Figure 21 Effect of method of collection of saliva on absorbance value. C only –A549 cells in saliva; S – saline; H – Hartmann's; S3/S10 – gargle with 3ml /10ml of saline plus A549cells; H3/H10 – gargle with 3ml / 10ml of Hartmann's solution plus A549 cells. The results are representative results showing the absorbance values from duplicate determinations in a single experiment.

3.3.3 Telomerase activity in patient saliva samples and controls

Patients newly diagnosed with HNSCC were recruited with ethical consent as previously described (n = 14). Saliva samples obtained were first of all assayed to

determine the protein concentration in each sample. Next, each sample was assessed for telomerase activity using the TRAP assay (TeloTAGGG Telomerase PCR ELISA kit - see materials and methods). Control samples (n= 13) were obtained and assayed similarly. For each patient sample, the maximum volume of lysate (25µl) that could be used in the PCR reaction was used. The concentration of protein in the 25µl volume of lysate varied between patients but was routinely between 0.002µg/µl and 0.34µg/µl (corresponding to a total amount of protein in the assay of 50ng and 8.5µg respectively), see figure 22 below.

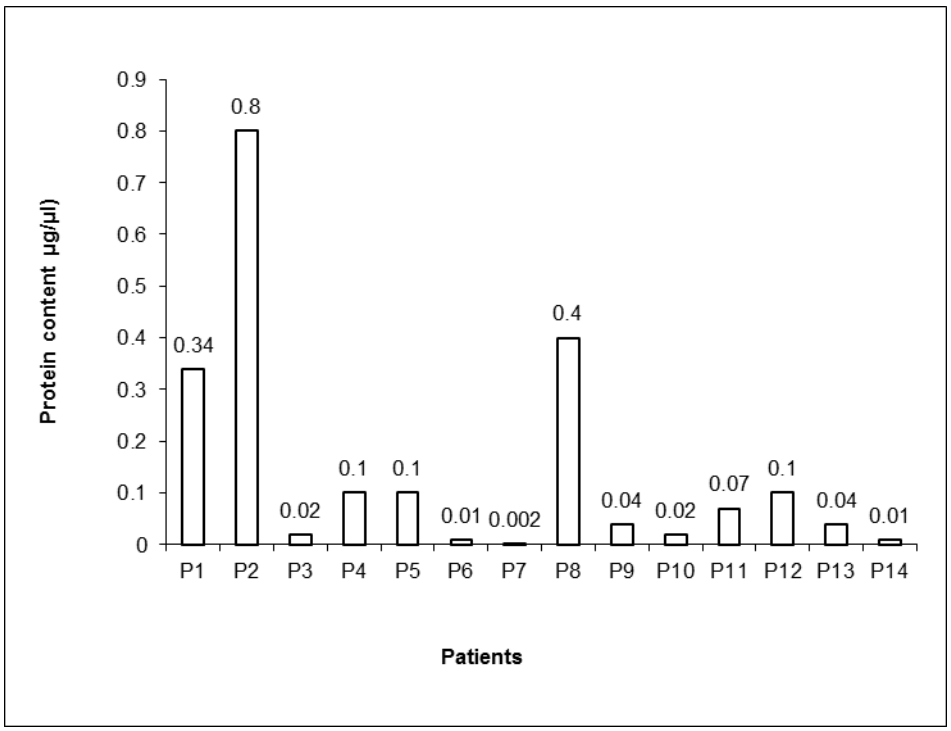


Figure 22 Protein concentration (µg/µl) of lysate for each patient (25µl of lysate was used in the PCR reaction).

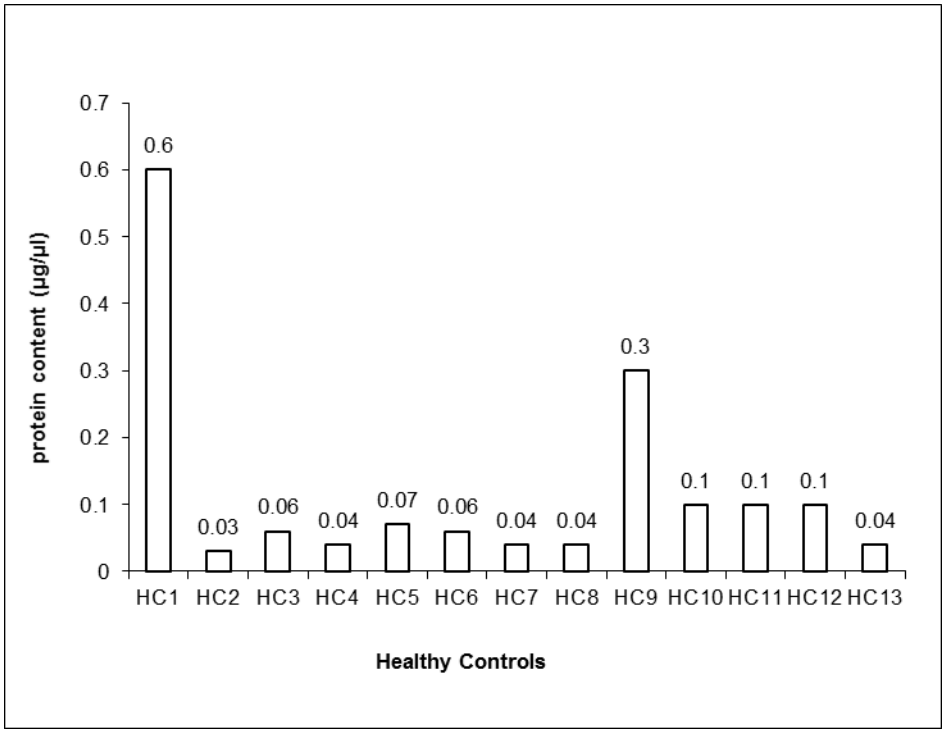
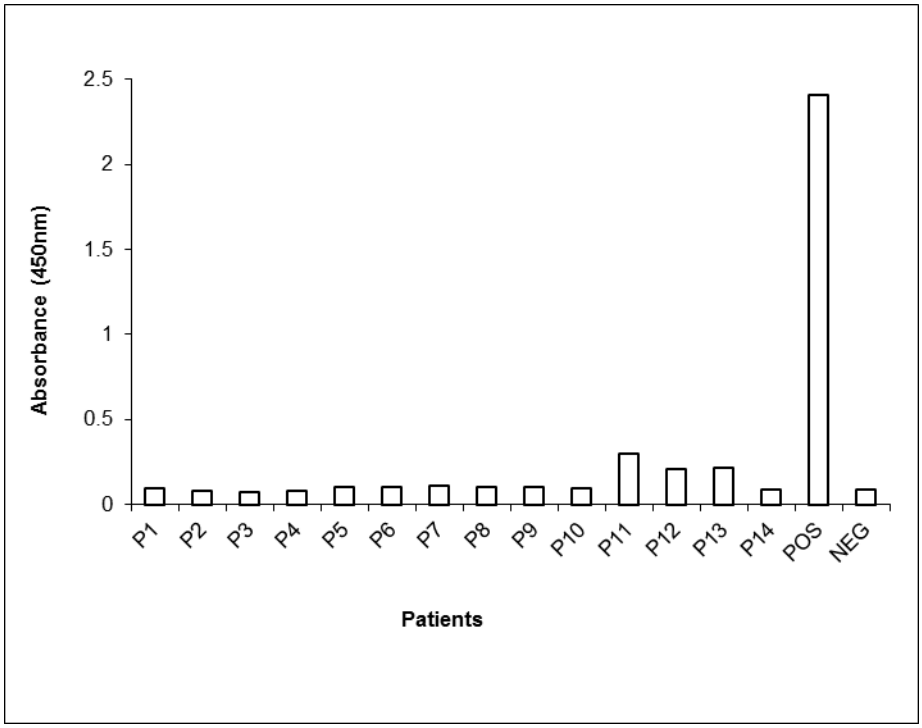


Figure 23 Protein concentration (µg/µl) of lysate for each Healthy control

As shown in figure 17, protein concentration as low as 0.02µg/µl (corresponding to total protein of 40ng), produced detectable telomerase activity and the protein concentration values obtained in some patients was above the amount of protein required to detect telomerase activity. However, the results obtained from patient samples show low absorbance values obtained in all samples. The results shown in figure 24 are typical of 3 other similar experiments using the same patient samples. There were no obvious differences between the two groups (patients and healthy controls).

A



B

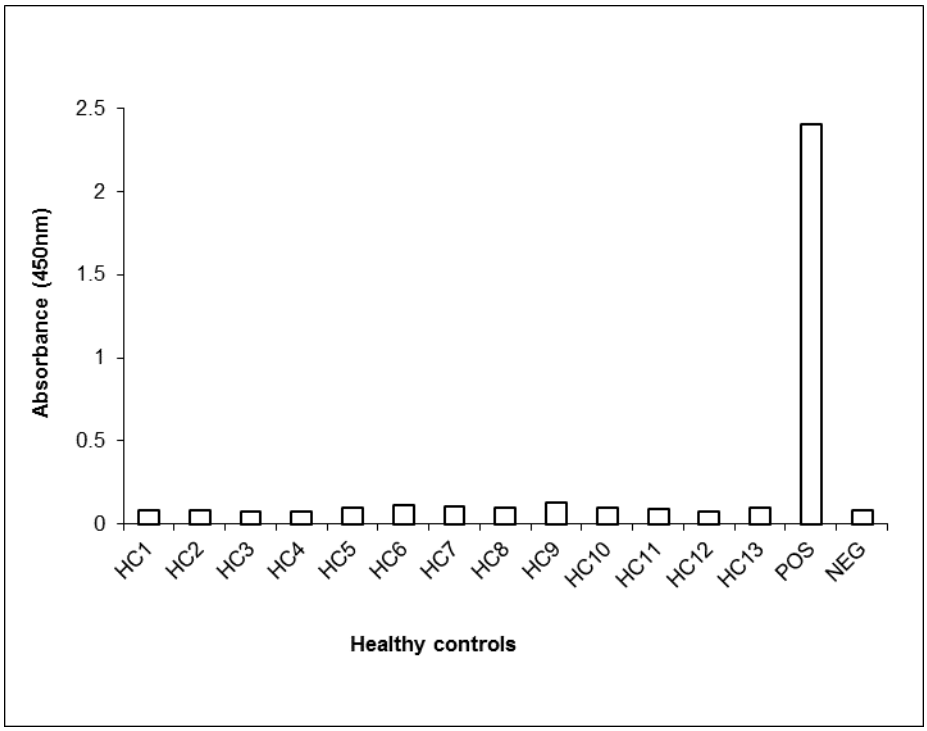


Figure 24 Telomerase activity in clinical samples. A – Samples from patients diagnosed with oral cancer (P - patient). B – Samples from healthy individuals of matched age and sex (HC – healthy controls).

3.3.4 Detection of telomerase hTERT protein expression in cells and tissue

First of all, hTERT expression was detected using immunocytochemistry in H157 cells – an oral squamous cell carcinoma cell line shown to be telomerase positive and in HUVEC cells shown to be telomerase negative. Telomerase expression was then determined in the patient tissue samples using immunohistochemistry.

Immunocytochemical detection of hTERT antibody

hTERT protein expression was determined in H157 (telomerase positive) and HUVEC cells (telomerase negative), see figure 53. H157 and HUVEC cells were cultured in 8 well chamber microscopic slides until they achieved 70% confluence. In order to determine the best concentration of antibody to use, different concentrations of hTERT antibody were produced by dilution with 1% goat serum (1:200, 1:100 and 1:50). Each concentration was applied to a pair of wells on the slides, and in the 4th pair of wells, the antibody was omitted in order to provide a negative control. Positive hTERT staining (heterogenous brown staining) was observed using the different dilutions of primary antibody in the H157 cells but not in the negative controls in which the antibody was omitted, indicating that there was hTERT protein expression in H157 cells (figures 25 and 26). The brown staining was most intense with 1:50 dilution of the antibody. There was no hTERT staining seen in the HUVEC cells or controls (Figure 27). From these series of experiments, 1:50 dilution of the antibody was selected as the primary antibody dilution used.

A

B

Figure 25 hTERT expression in H157 cells. A 1:100 dilution of antibody. B 1:50 dilution of primary antibody. Taken at 20x objective lens magnification

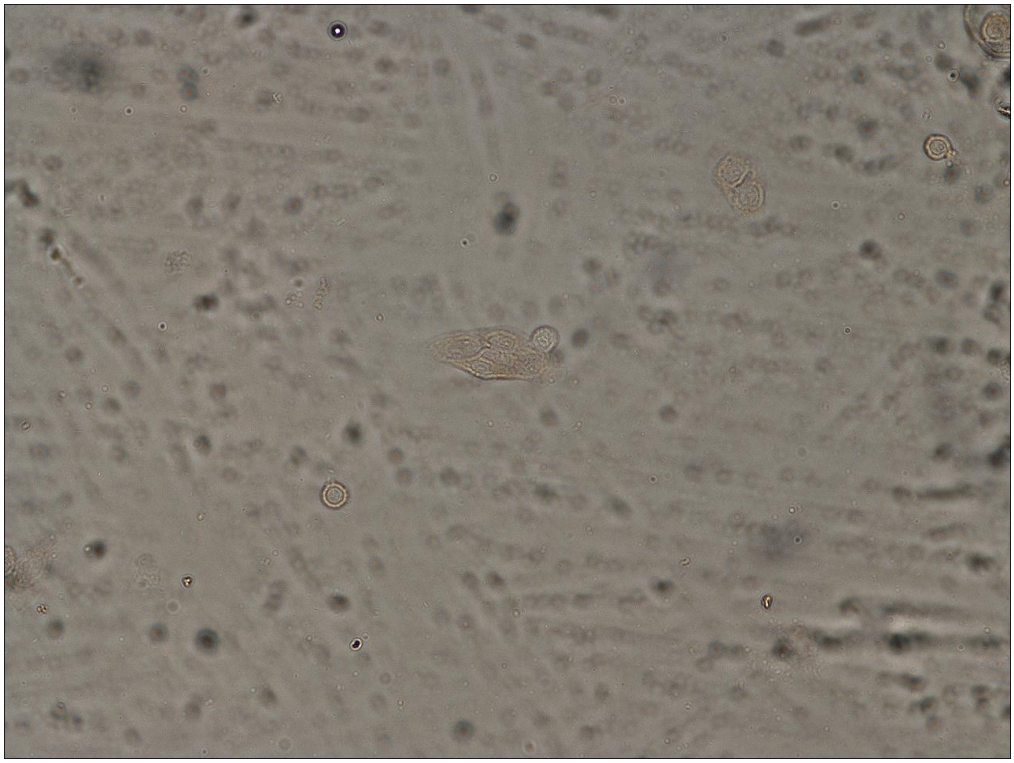
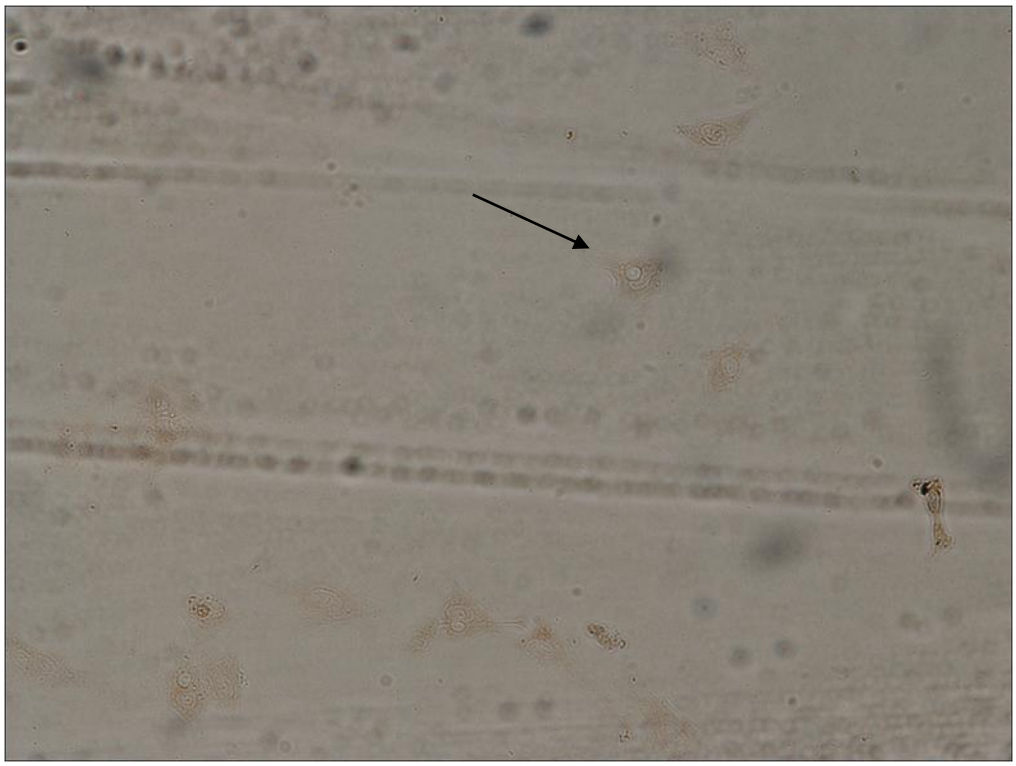


Figure 26 Absence of hTERT expression in H157 cells in the negative controls in which the antibody was omitted. Taken at 20x objective lens magnification

A



B

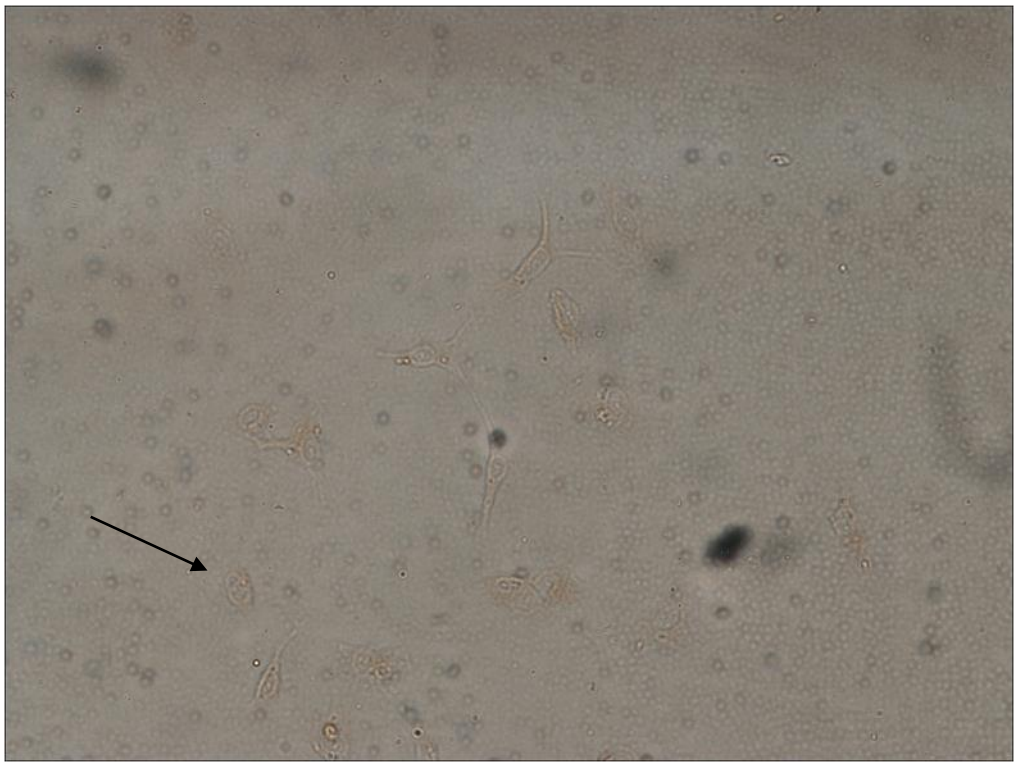


Figure 27 Absence of hTERT expression in A - HUVEC cells with 1:50 dilution of primary antibody and B – HUVEC cells negative controls. Taken at 20x objective lens magnification

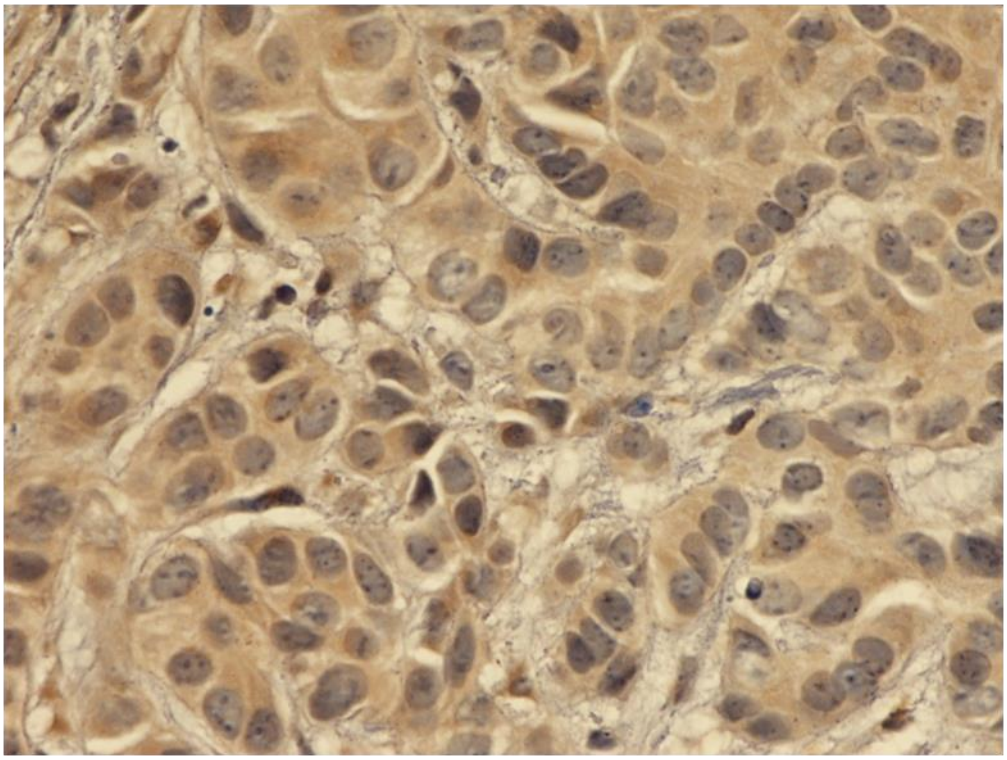
Immunohistochemical detection of hTERT antibody

A549 xenografts were derived from subcutaneously grown tumours in NCR/Nu mice, as described in materials and methods (sections 2 and 3). Patient tissue samples were obtained with ethical consent from some patients in the same group as in the saliva study (not all the patients had tissue samples available). Expression of hTERT was initially determined and validated in xenograft tissue derived from the A549 cells.

Determination of best antigen retrieval method

In order to optimize the experimental technique, two different antigen retrieval methods were compared: one using the microwave and another with the oven. Both methods were performed using different concentrations of primary antibody as well as no antibody - negative control, (see materials and methods). Using the microwave technique, the slides were immersed in antigen retrieval solution and heated in the microwave for 20 min or 10 min and one set of slides were not heated the microwave. Using the oven technique, the slides were immersed in the antigen retrieval solution, and then placed in the oven at 90^{o}C for 40 min. The results are shown in figures 28 to 34 below. Results show heterogenous brown staining in the nuclei and cytoplasm in the xenografts, with increasing intensity of staining using the increasing primary antibody concentrations. There was no staining in the negative controls which had no antibody. There was no difference in the intensity of staining using the different methods of antigen retrieval. The microwave technique involving heating the slides for 20 minutes was therefore used for further experiments. Based on the good intensity of staining using 1:50 dilution of the primary antibody, this was selected as the antibody dilution used for further studies.

A



B

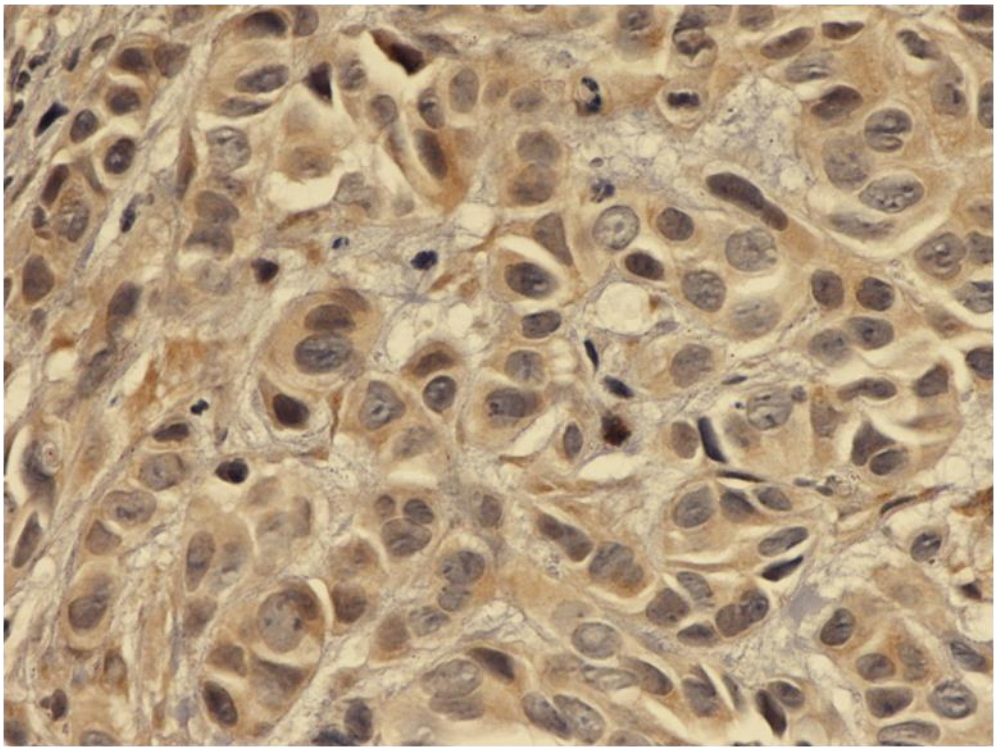
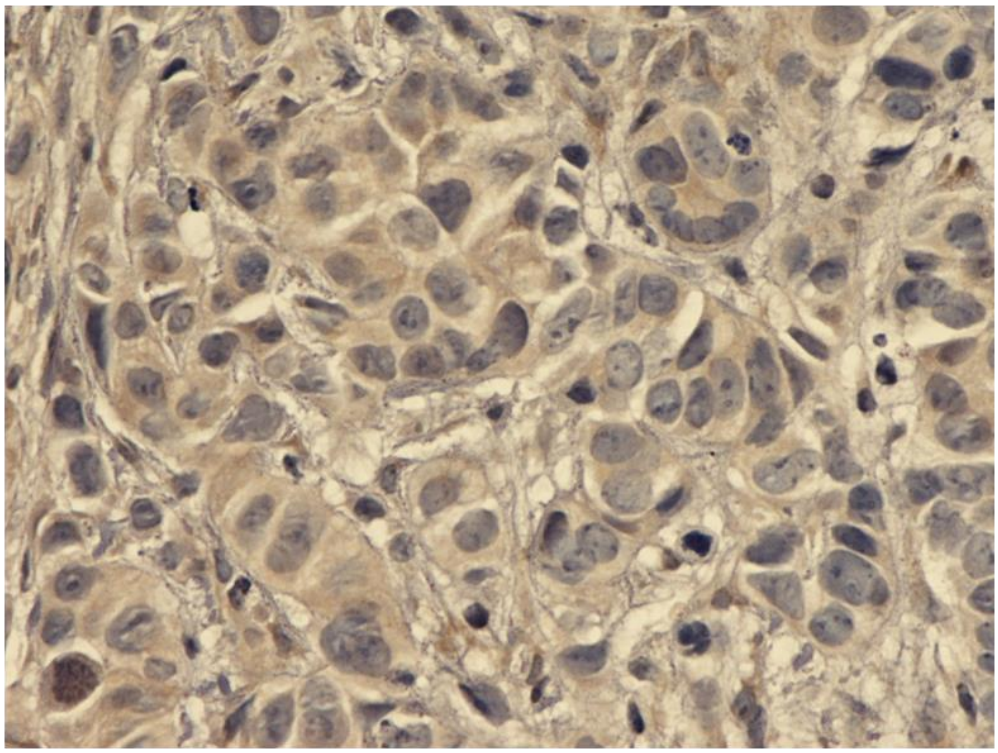


Figure 28 hTERT expression in A549 xenograft using the microwave antigen retrieval technique (10 min microwave): A 1:50 primary antibody dilution, B 1:100 primary antibody dilution. Taken at 20x objective lens magnification.

A



B

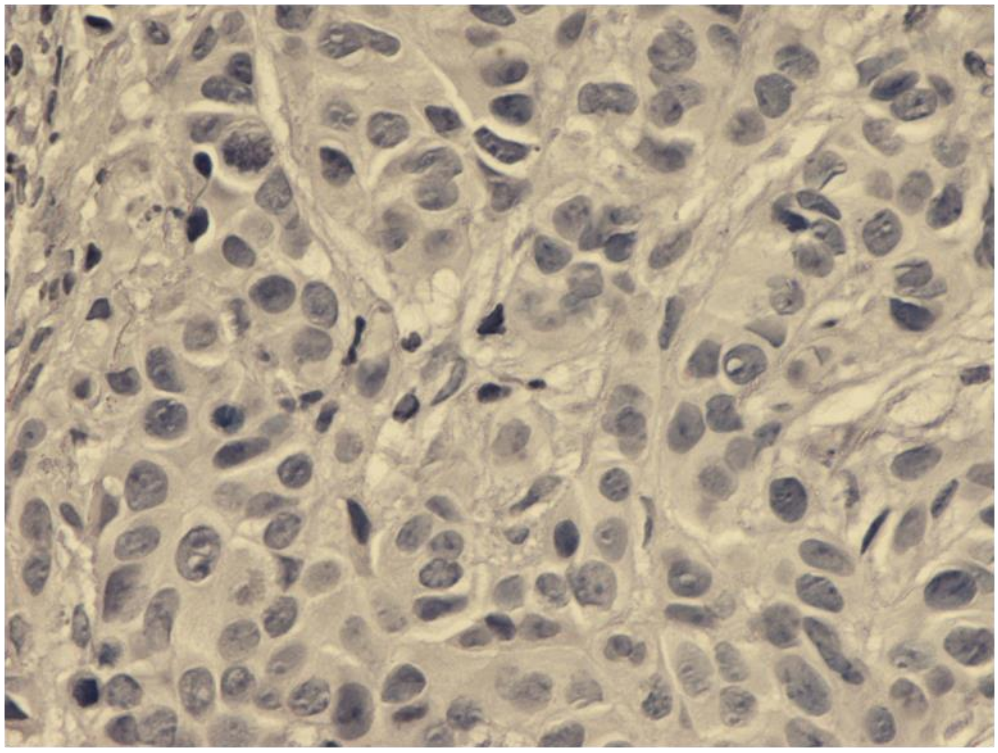
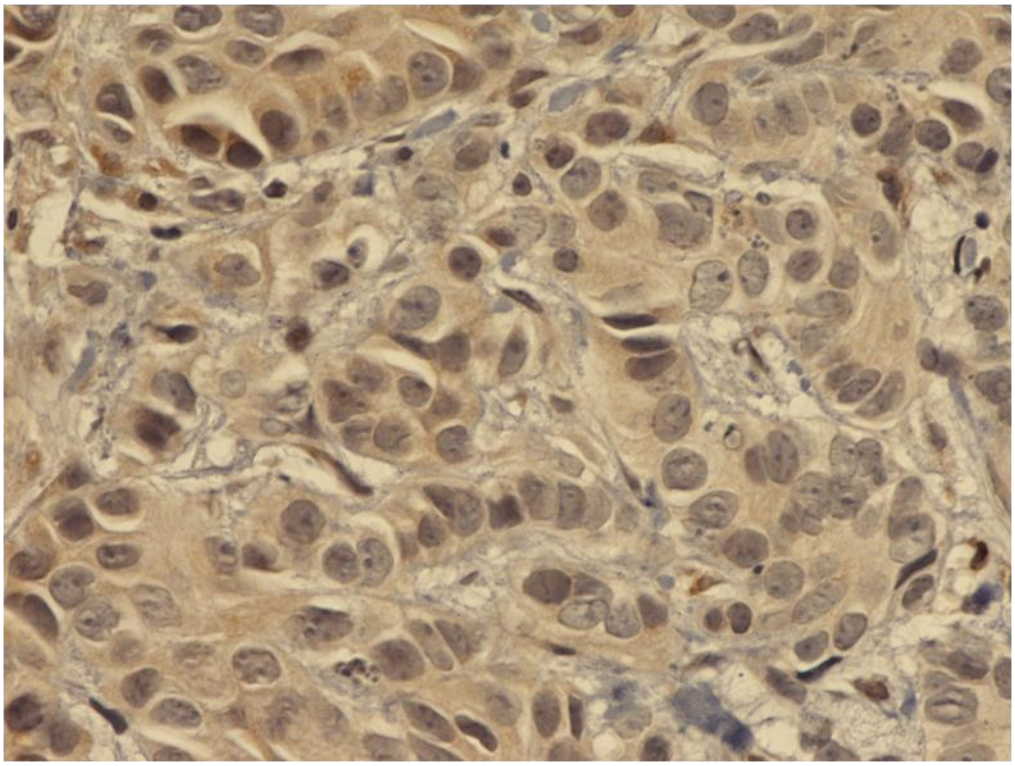


Figure 29 hTERT expression in A549 xenograft using the microwave antigen retrieval technique – (10 min microwave): A 1:200 primary antibody dilution, B negative control. Taken at 20x objective lens magnification

A



B

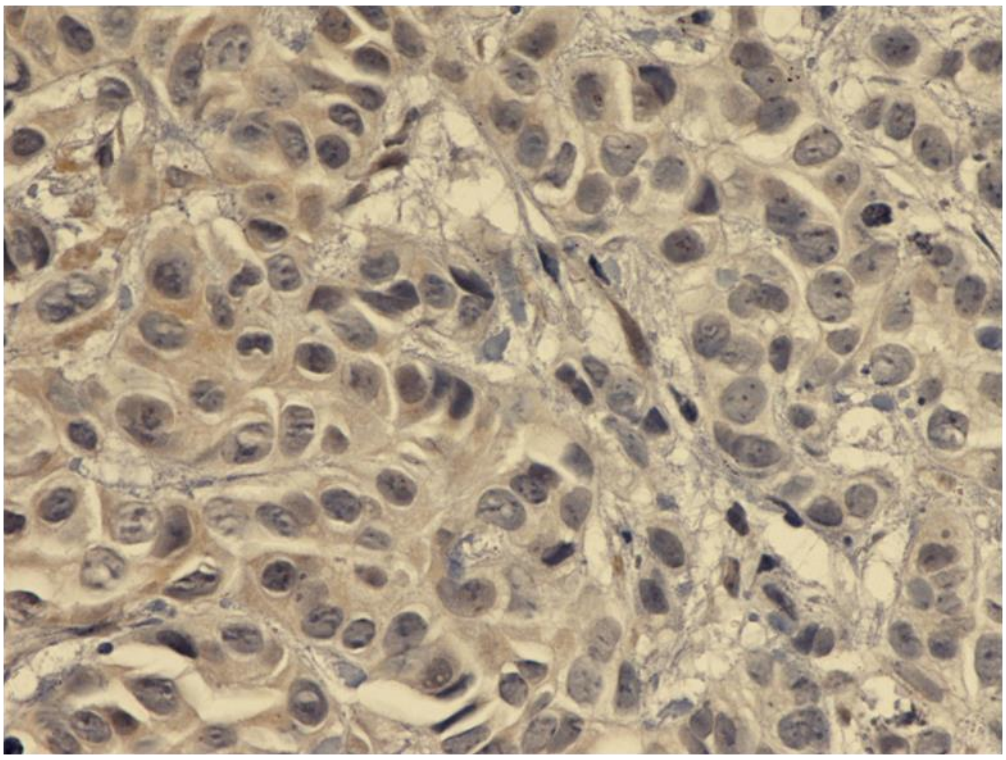
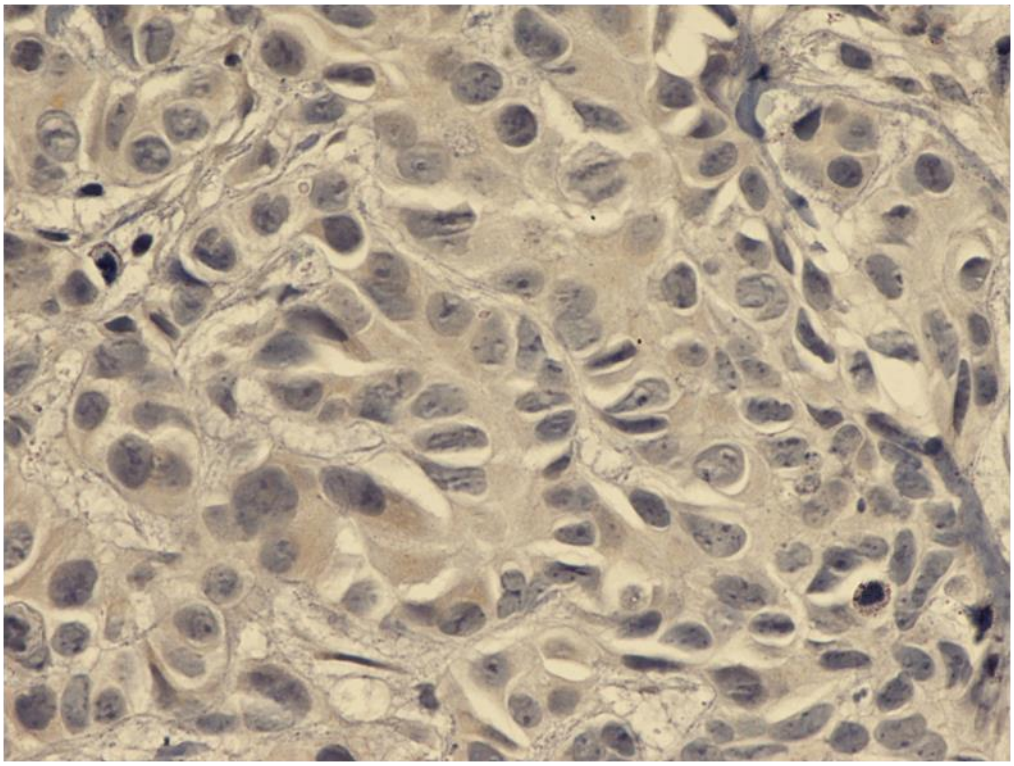


Figure 30 hTERT expression in A549 xenograft using the microwave antigen retrieval technique (20 min microwave): A 1:50 primary antibody dilution, B 1:100 primary antibody dilution. Taken at 20x objective lens magnification

A



B

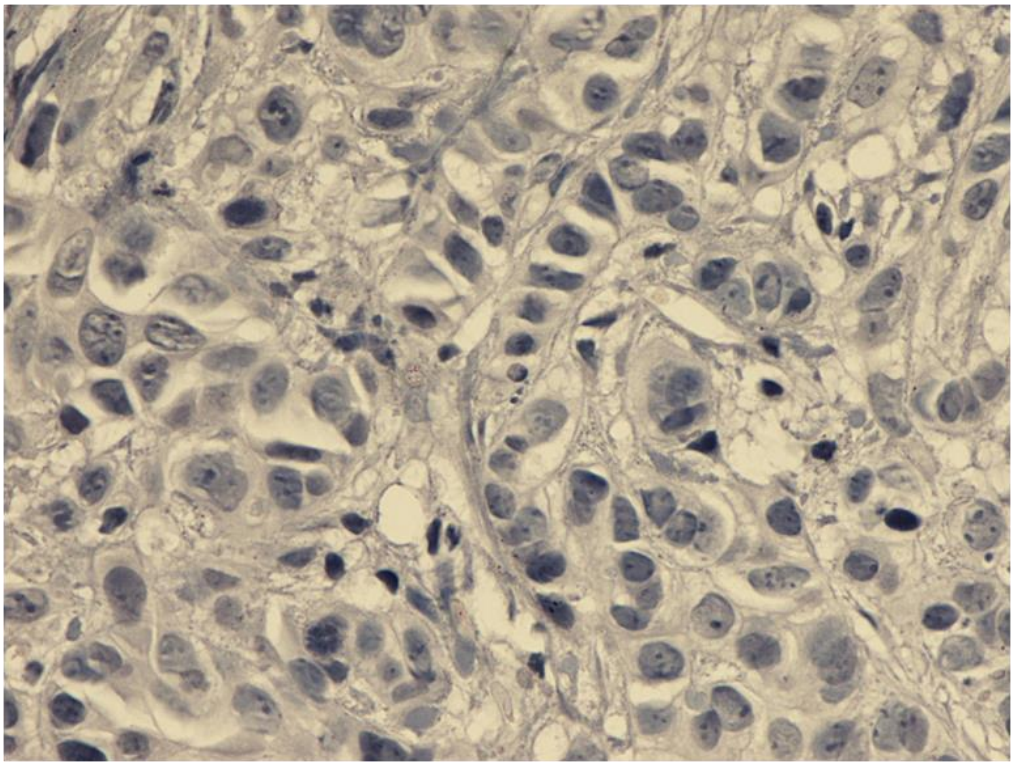
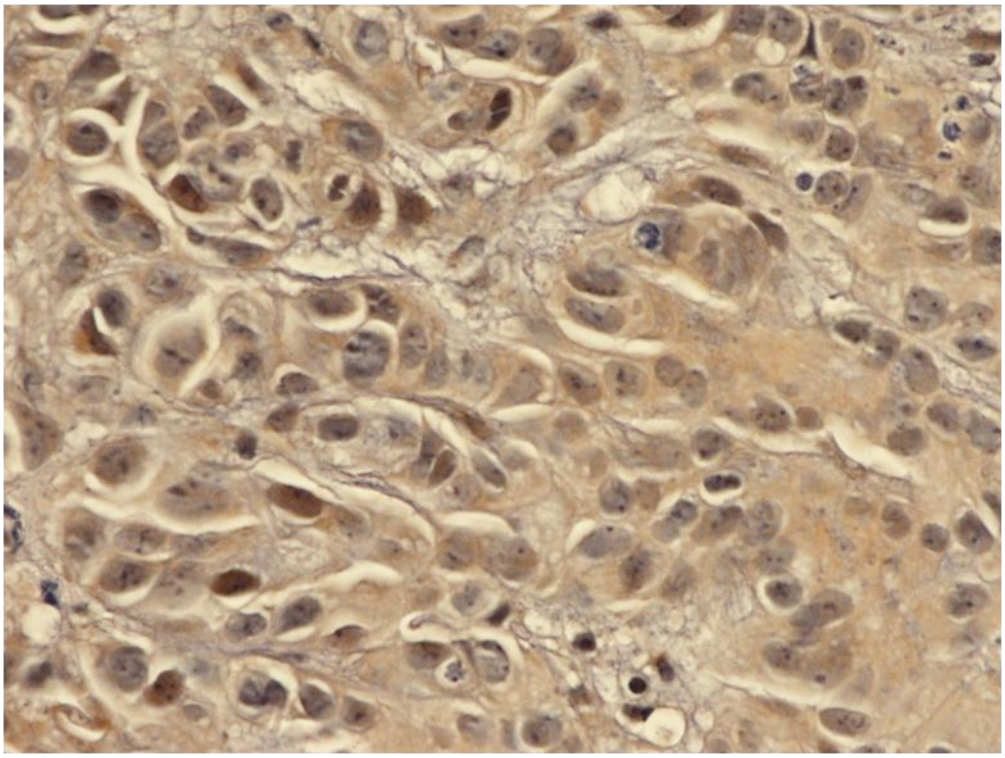


Figure 31 hTERT expression in A549 xenograft using the microwave antigen retrieval technique - 20min microwave: A 1:200 primary antibody dilution, B negative control. Taken at 20x objective lens magnification

A



B

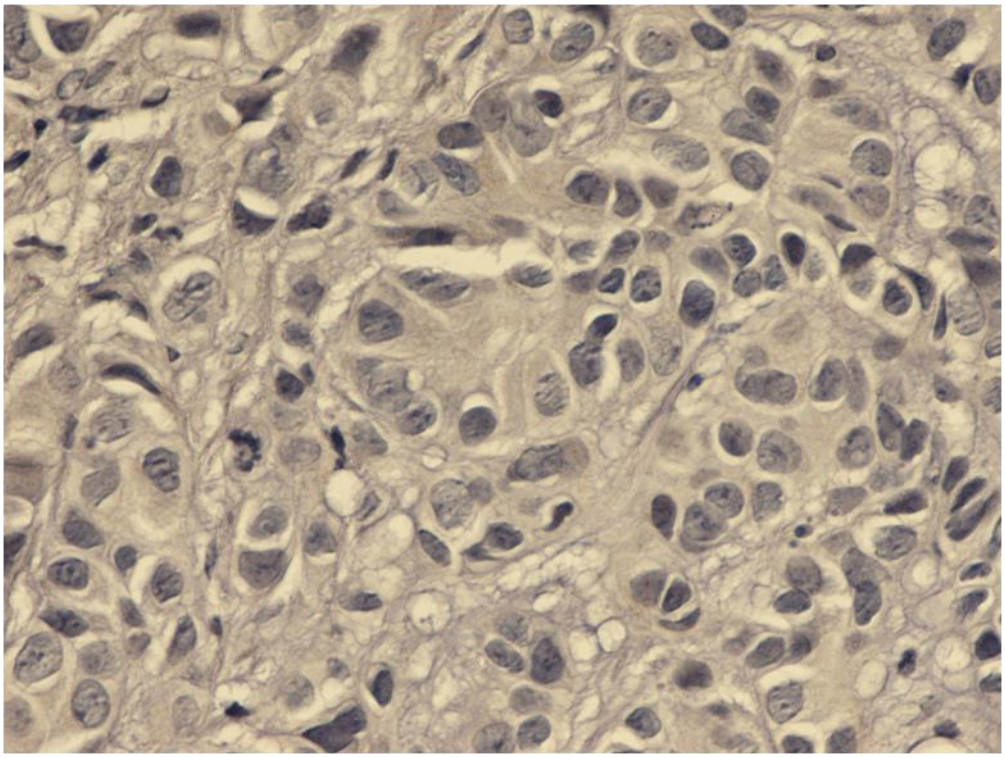
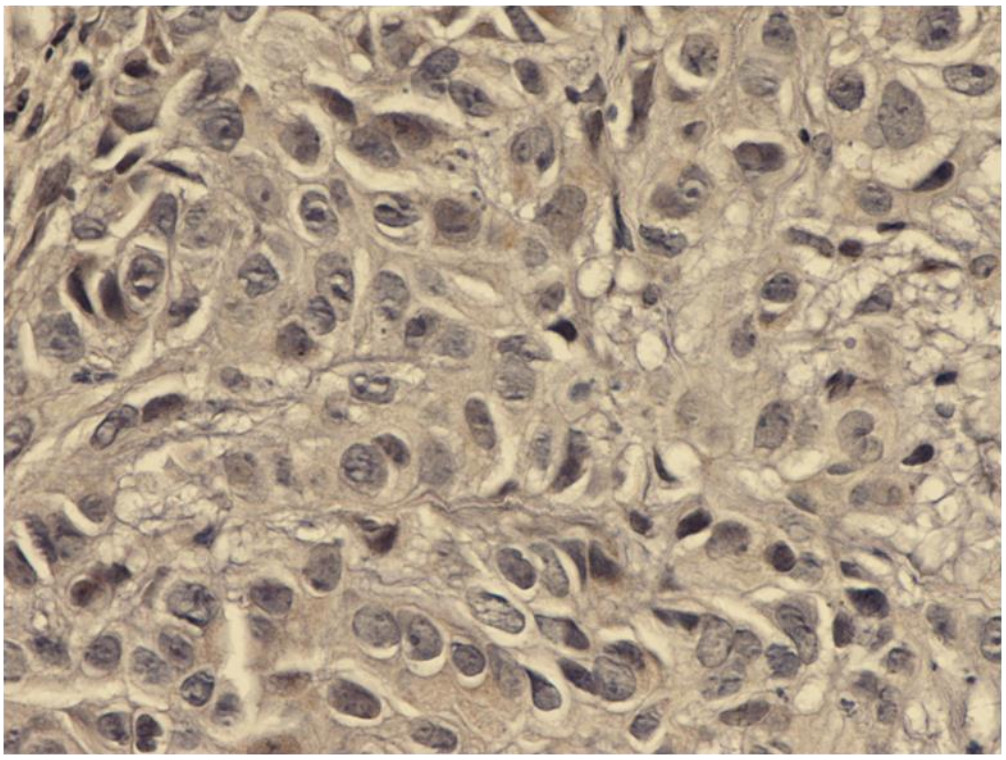


Figure 32 hTERT expression in A549 xenograft using the microwave antigen retrieval technique (no microwave): A 1:50 primary antibody dilution, B 1:100 primary antibody dilution. Taken at 20x objective lens magnification.

A



B

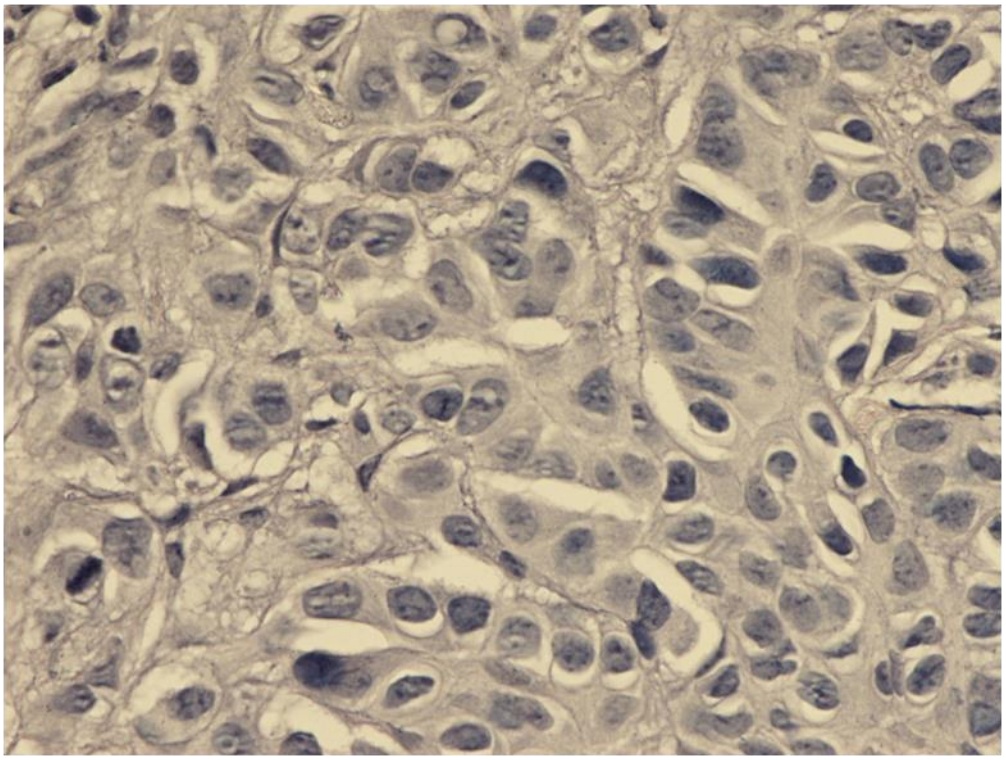
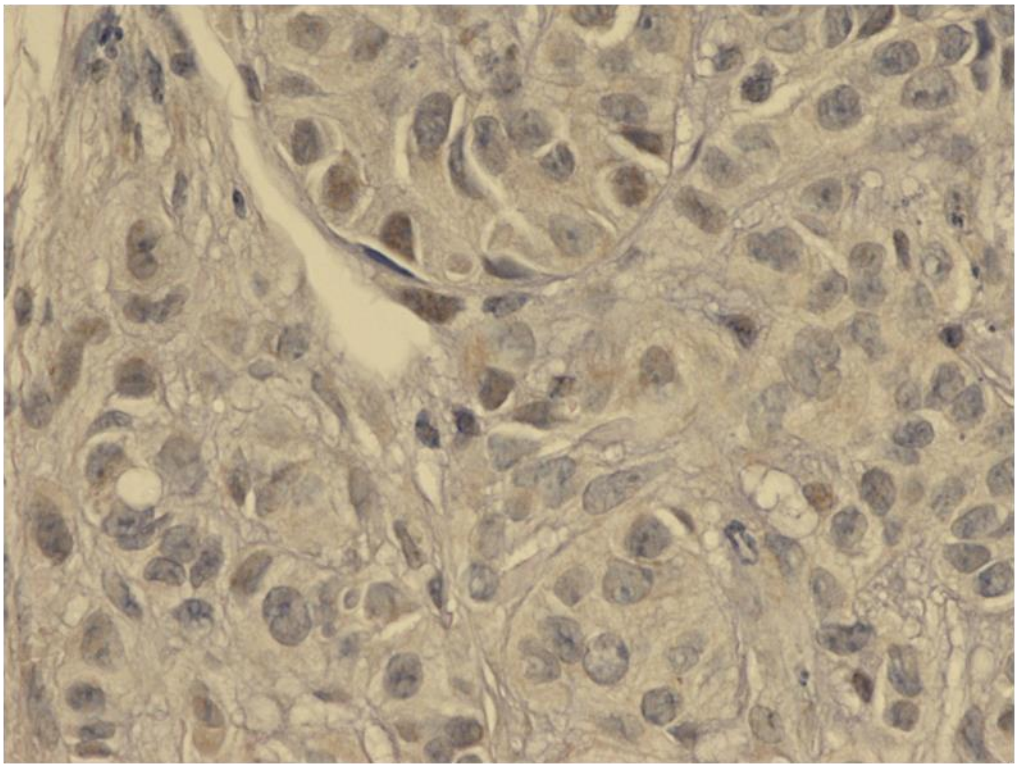


Figure 33 hTERT expression in A549 xenograft using the microwave antigen retrieval technique (no microwave): A 1:200 primary antibody dilution, B negative control. Taken at 20x objective lens magnification.

A



B

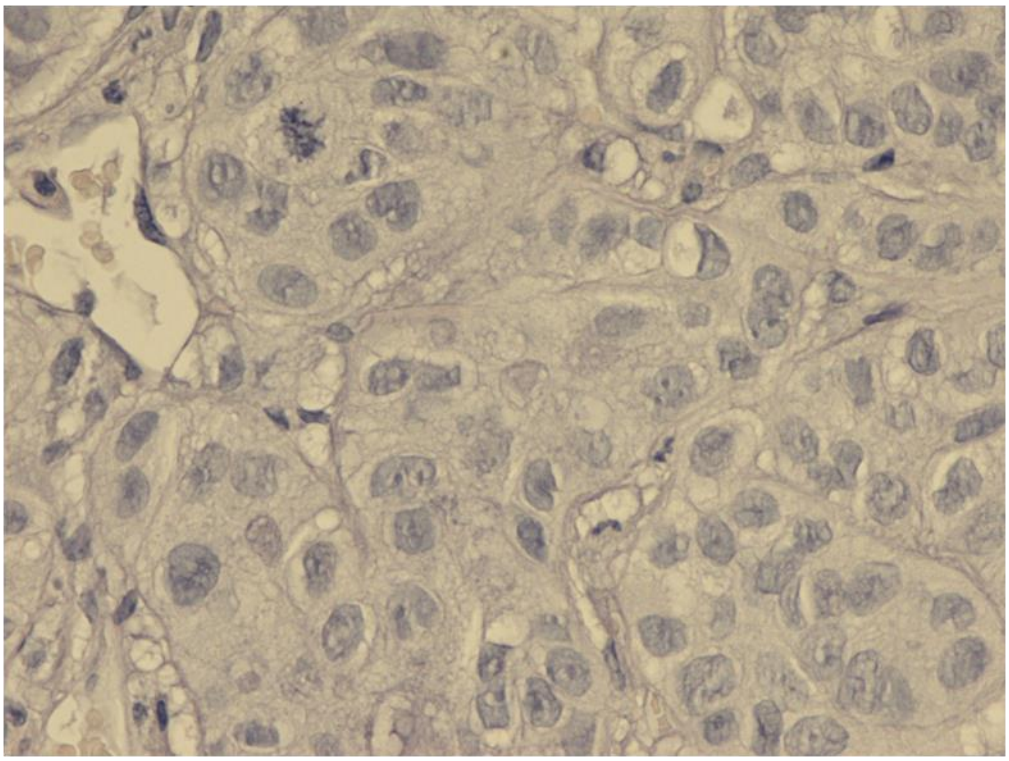


Figure 34 hTERT expression in A549 xenograft using the oven method – A 1:50 dilution of primary antibody, B negative control. Taken at 20x objective lens magnification

3.3.5 Expression of hTERT in patient tissue and controls

Next, hTERT expression was determined in patient tissue samples and from normal tonsillar tissue which served as controls. As previously mentioned, tissue samples were not available for all patients; therefore results below are a representative sample of the results obtained in 3 patients. A 1:50 dilution of primary antibody was used and antigen retrieval was performed by immersing the slides in antigen retrieval solution and heating the slides for 20 minutes in the microwave (as described in section 3.2.8). Patient tissues expressed heterogenous brown staining indicating hTERT expression, whilst the negative controls (without the primary antibody added), and normal tonsillar tissue did not show brown staining (figures 35 – 38).

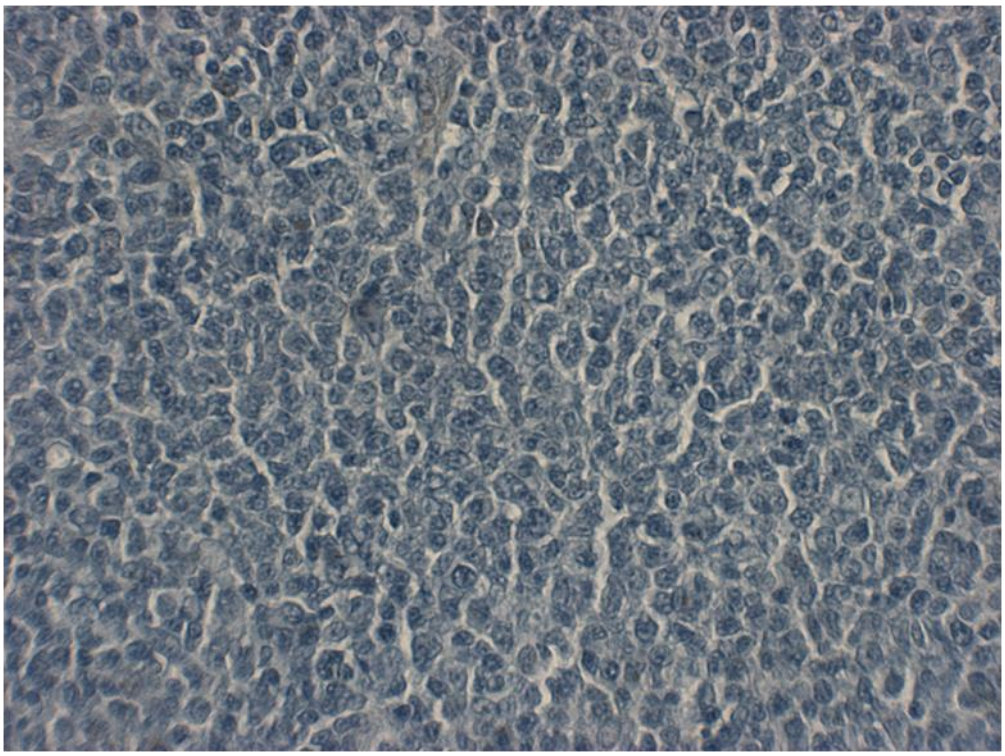
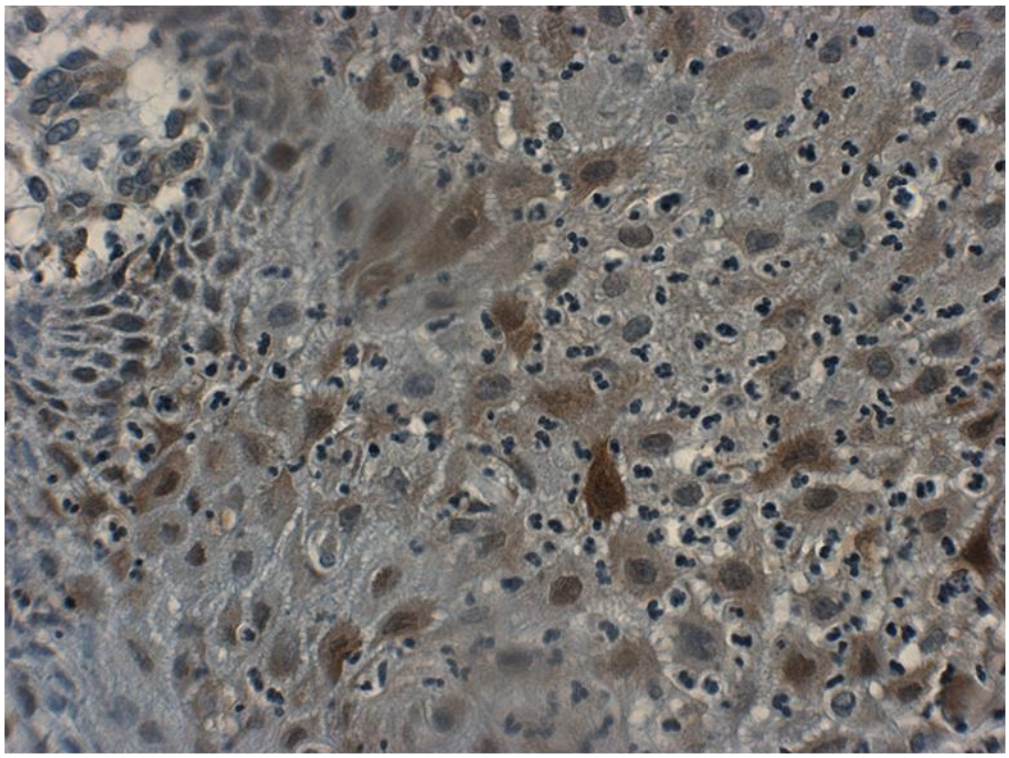


Figure 35 Absence of hTERT expression in normal human tonsillar tissue using 1:50 dilution of primary antibody. Taken at 20x objective lens magnification.

A



B

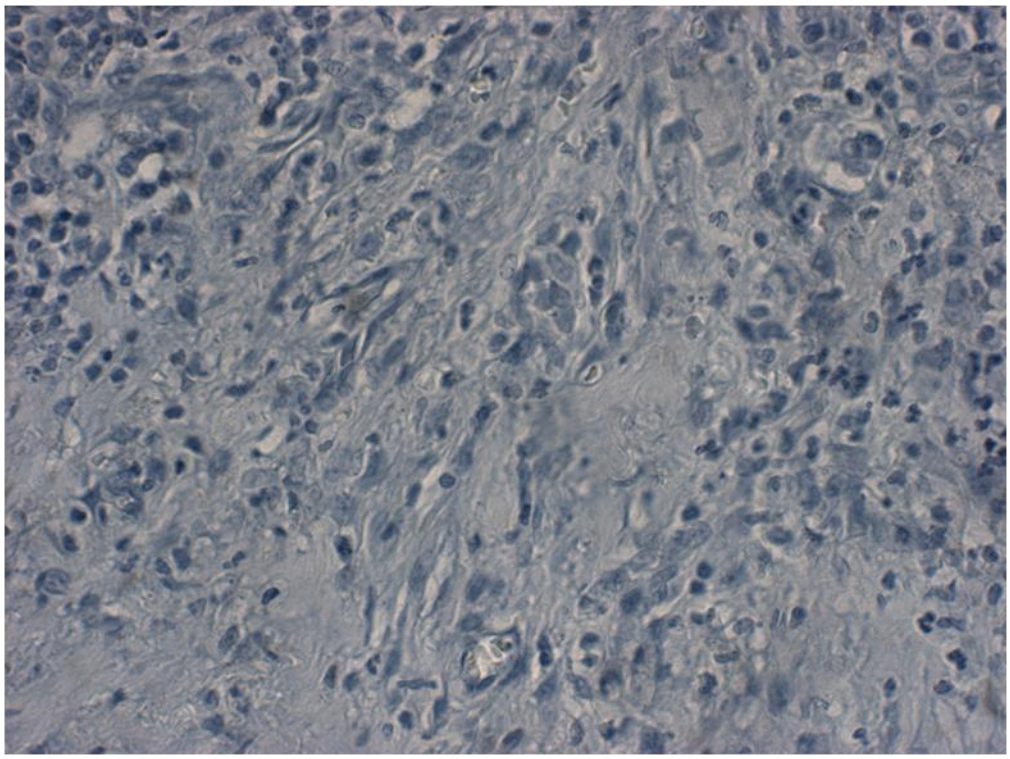
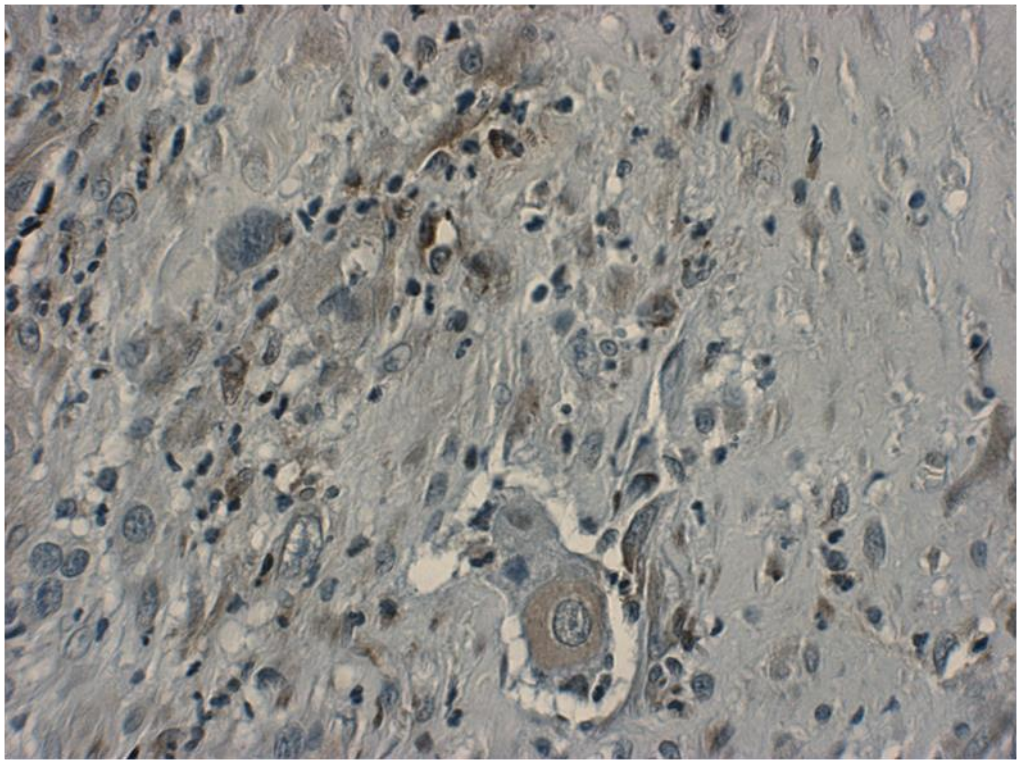


Figure 36 hTERT expression in patient tissue sample – patient 1. A - using 1:50 dilution of primary antibody, B - negative control. Taken at 20x objective lens magnification.

A



B

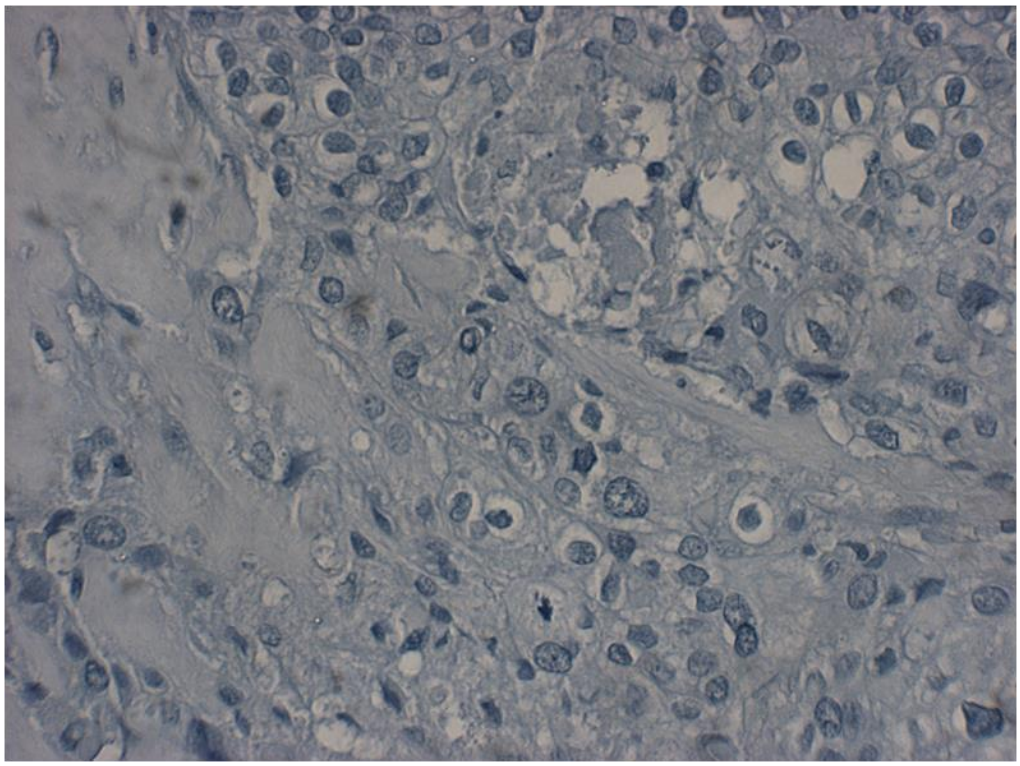
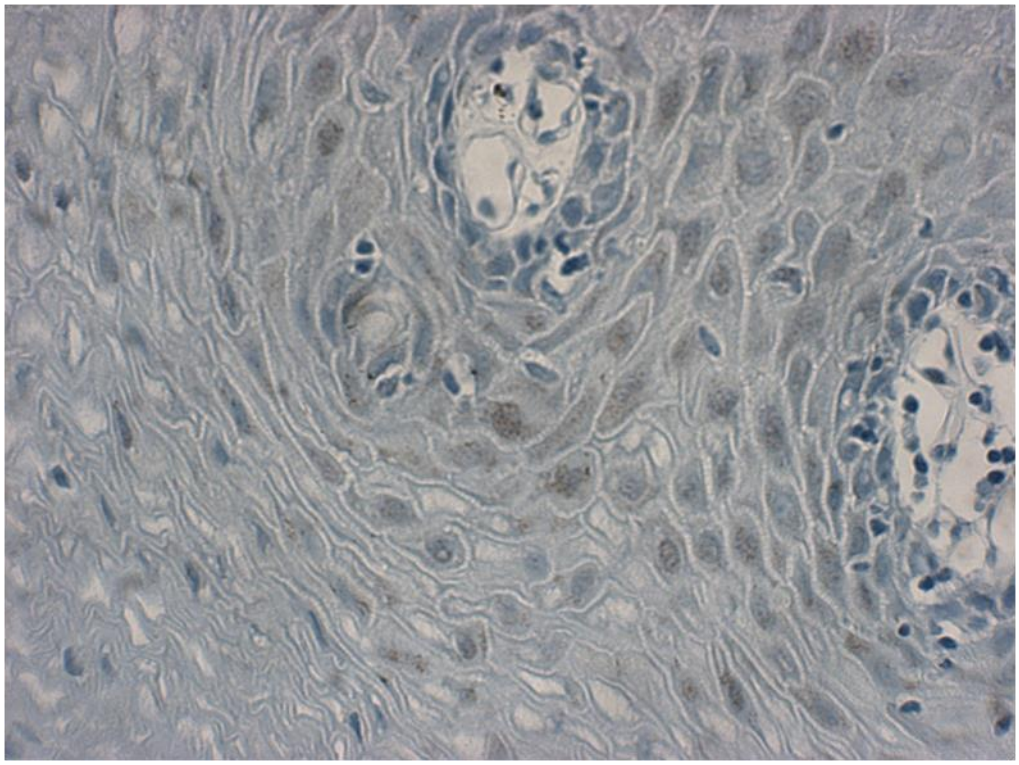


Figure 37 hTERT expression in patient tissue sample – patient 2. A - using 1:50 dilution of primary antibody, B - negative control. Taken at 20x objective lens magnification.

A



B

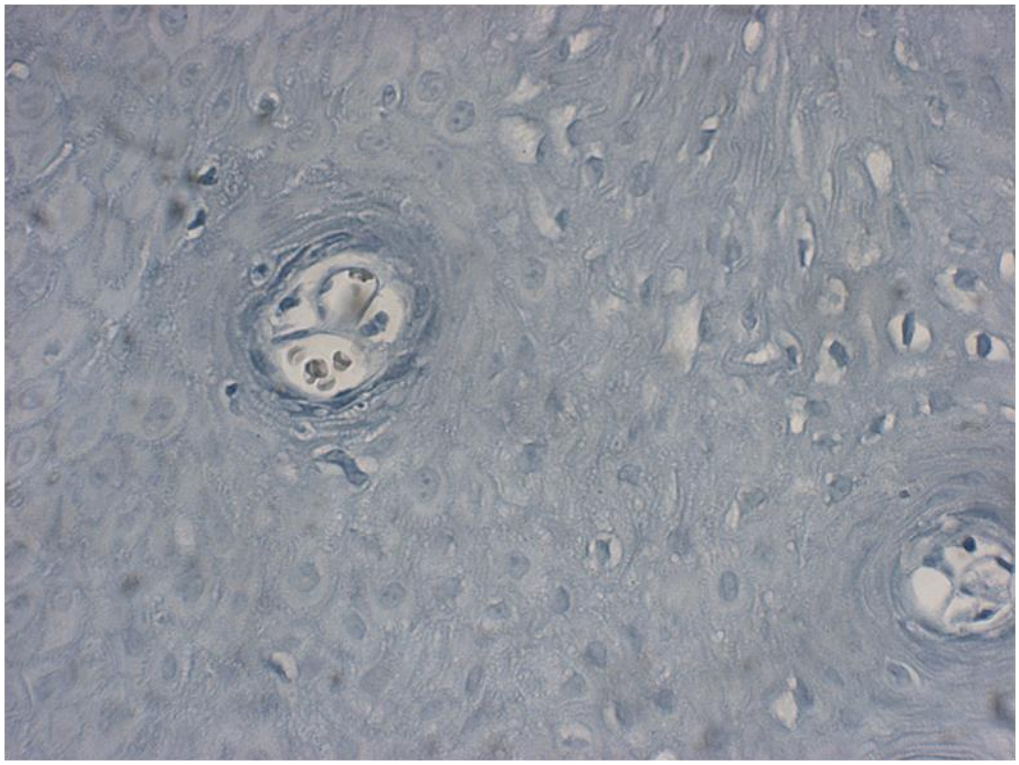


Figure 38 hTERT expression in patient tissue sample – patient 3. A - using 1:50 dilution of primary antibody, B - negative control. Taken at 20x objective lens magnification.

3.4 Telomerase inhibition

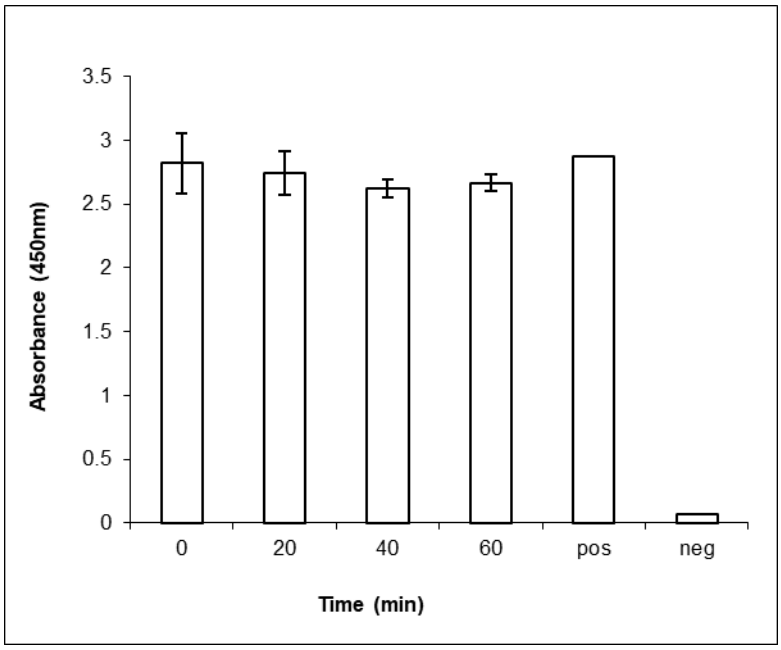
In parallel with studies to investigate telomerase in tissue and saliva samples from HNSCC patients, studies were also undertaken to characterize compounds with putative telomerase inhibitory activity. The compounds were synthesized at the School of Pharmacy, University of Bradford.

In these series of experiments, a methodology for determining the activity of novel compounds on telomerase activity was derived by first assessing the stability of the enzyme at experimental temperatures (37^0C) over time and also determining the effect of the compounds solvent (DMSO) on the TRAP reaction. In order to assess the direct effects of the compounds on telomerase activity, A549 cells lysates were incubated with the compounds for varying lengths of time and then the remaining telomerase activity was measured.

3.4.1 Stability of telomerase at 37^0C with time

To determine the stability of telomerase in the cell lysates, A549 cell lysates were incubated at 37^0C for different time periods (20 min to 240 min). The results in figure 39 showed that the enzyme was stable for up to 120 min (2h). After 120 min, the telomerase activity (represented by the absorbance values) began to decline.

A



B

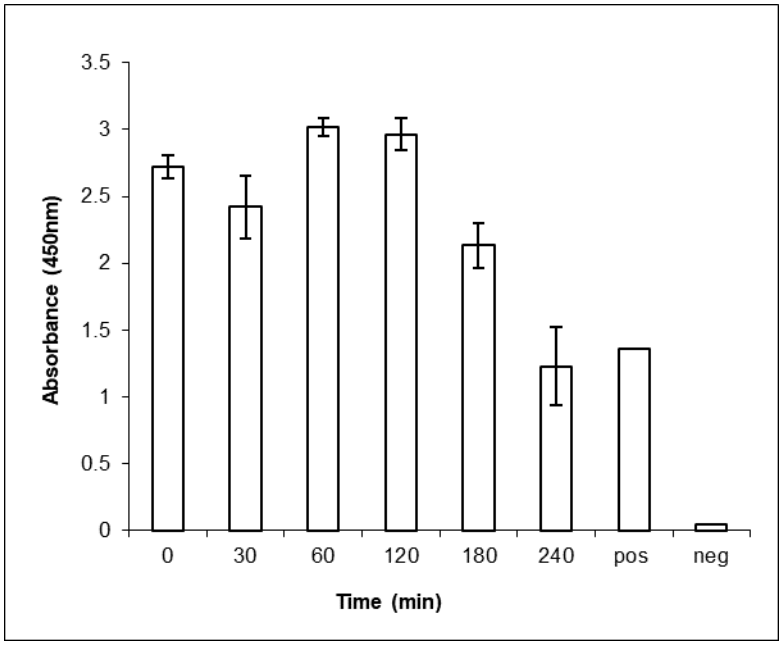


Figure 39 Stability of telomerase enzyme at 37^0C with time.
A, incubation of A549 cell lysate at 37^0C from 0 min (no incubation) to 60 min. B, incubation at 37^0C from 0 min to 240 min. The results are representative results showing the absorbance values from duplicate determinations in single experiments.

3.4.2 The effect of DMSO on the TRAP reaction

DMSO was used as solvent for a few of the compounds screened. The effect of DMSO on the TRAP reaction within the telomerase PCR ELISA kit was therefore assessed by adding 100% DMSO to aliquots of A459 cell lysate (produced from $2x10^5$ cells) to make final DMSO concentrations of 5, 2.5, 1, 0.5, 0.25 and 0.1% (v/v). The lysates were then immediately assayed for their telomerase activity. The results are shown in figure 40. There appeared to be no change in the absorbance values obtained in the presence of different concentrations of DMSO, suggesting that DMSO did not affect telomerase activity. The concentration of DMSO subsequently used in the experiments was typically 0.1% however.

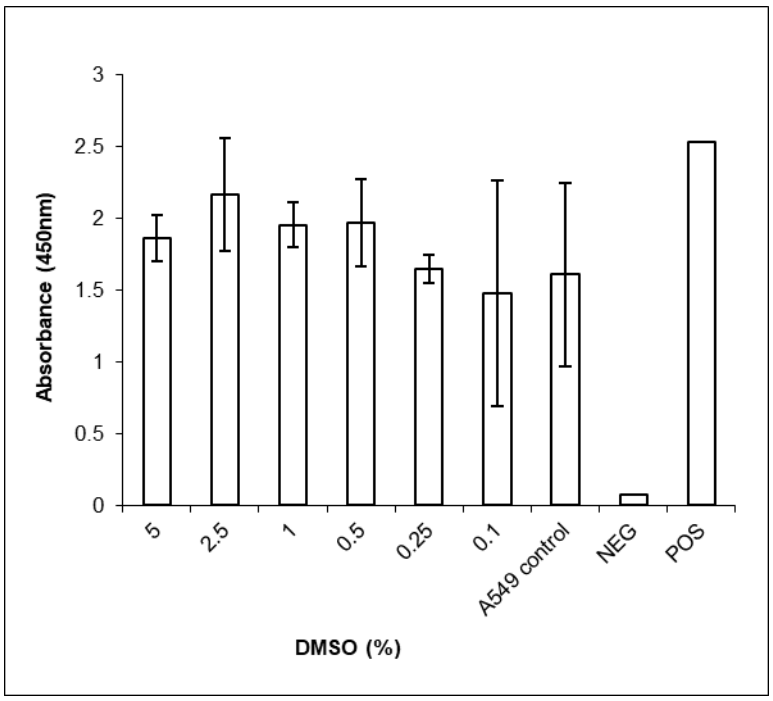


Figure 40 Effect of DMSO on the telomerase activity in A549 cell lysates. (A549 control - A549 cell lysate without DMSO). The results are representative results showing the absorbance values from duplicate determinations in a single experiment.

3.4.3 Effect of known cytotoxic agents on telomerase activity

In order to determine the effect of some established cytotoxic agents on telomerase activity, 5 fluorouracil, cisplatin and doxorubicin were selected for testing. An initial stock concentration of 1mM of each agent was prepared. This was added to A549 cell lysates (prepared from 2 x 10^5 cells) to make final concentrations of 200µM which were then incubated at 37^0C for 2h. The results are shown in figure 41. The results show complete inhibition of telomerase activity by doxorubicin and partial inhibition by 5 fluorouracil, but no inhibition by cisplatin.

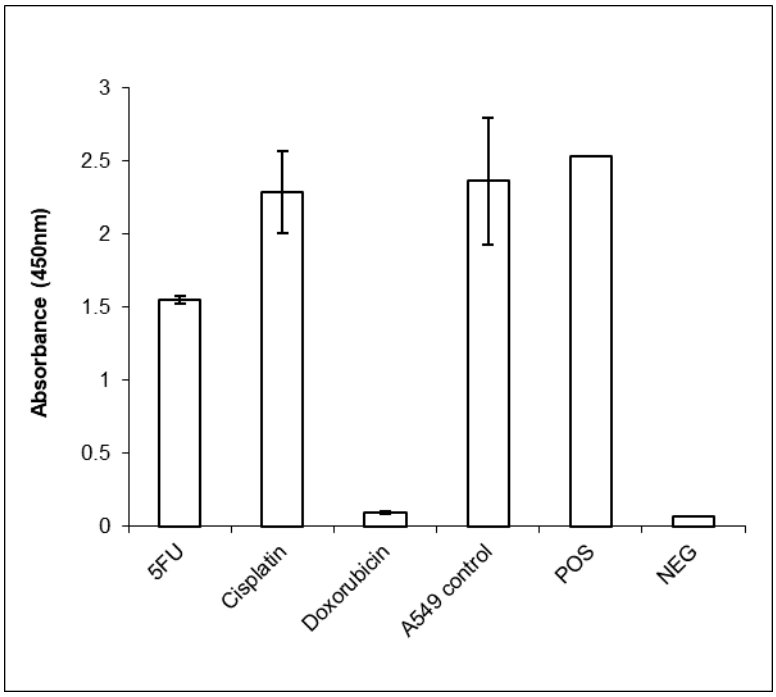


Figure 41 Effect of 5FU, doxorubicin and cisplatin on telomerase activity in A549 cell lysates. The results show the mean absorbance values from duplicate determinations in a single experiment

3.4.4 Berberine chloride as a positive control

Berberine chloride was selected as a positive control to develop an experimental technique for screening novel compounds (it was shown to be a moderate inhibitor of telomerase by Naasani et al in 1999). In order to assess the ability of berberine chloride to inhibit telomerase activity under the assay conditions used to screen the compounds, berberine chloride in aqueous solution was added to aliquots of A549 cell lysate (produced from $2x10^5$ cells). Final concentrations of 200, 20, 2 and 0.2µM were produced. The control was A549 cell lysate (termed A549 control) plus a volume of water equal to the drug volume was used. The effect of adding berberine chloride to cell lysates without incubation, and then addition of 200µM of berberine chloride over a time period of 30, 60 and 120min was first determined. Berberine chloride did not produce an immediate inhibition of telomerase activity when it was not incubated with the lysates as shown in figure 42. However, after incubation of the cell lysates over time with 200µM of berberine chloride, absorbance values were reduced (figure 43). This reduction in telomerase activity was most marked at 120min (2h). Based on this, the effect of different concentrations of berberine chloride on the cell lysates after 2h incubation was then determined (figure 44). The maximum inhibitory activity of berberine chloride occurred at 200µM.

From this series of experiments, 2h was selected as the exposure time of the cell lysates to the compounds as it was shown that the enzyme remained stable for up to 2h at 37^0C in figure 39, and also the reduction in telomerase activity after exposure to 200µM of berberine chloride over a time period was most marked at 2h.

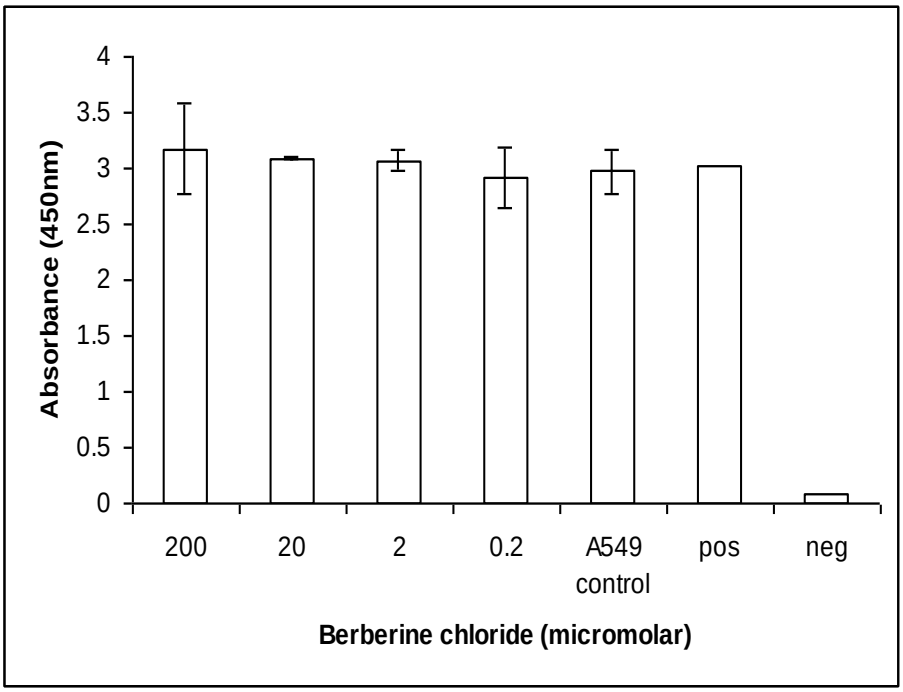


Figure 42 Effect of berberine chloride on telomerase activity in A549 cell lysates without incubation. The results are representative results showing the absorbance values from duplicate determinations in a single experiment.

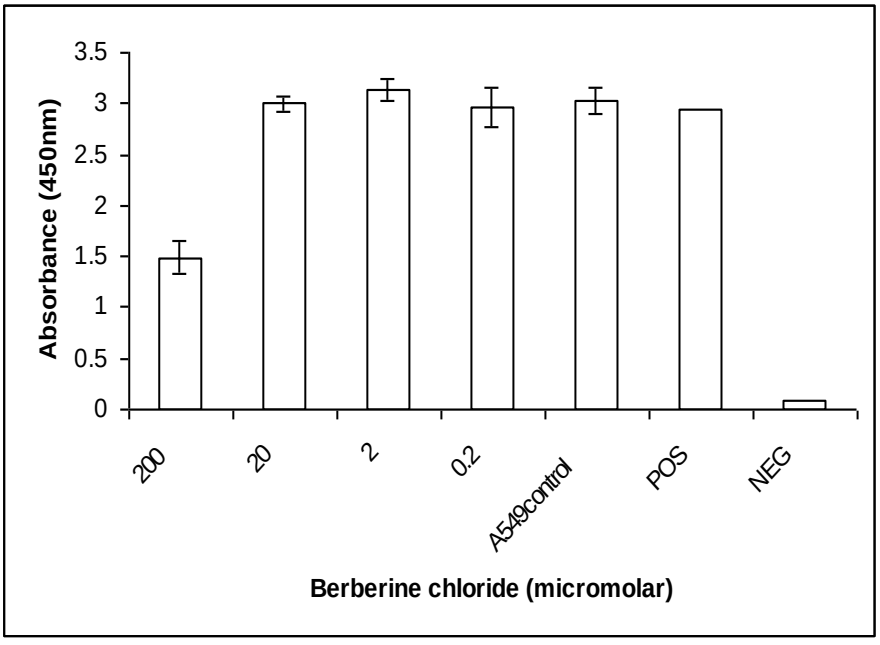


Figure 43 Effect of 200µM of berberine chloride on A549 cell lysates over a time period compared to lysates not exposed to berberine chloride. The results show mean absorbance values from 4 determinations in a single experiment

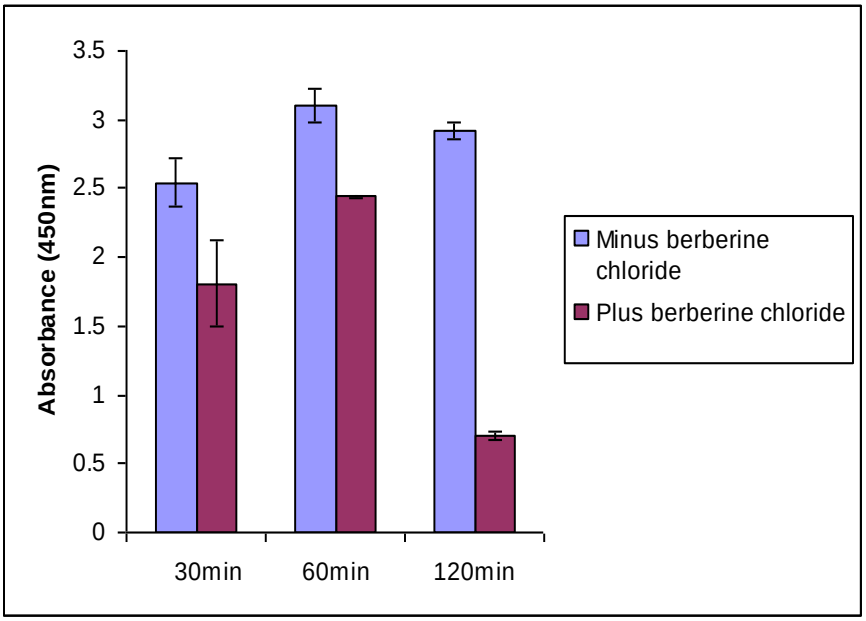


Figure 44 Effect of a range of concentrations of berberine chloride on telomerase activity in A549 cell lysates following 2h incubation. The results show the mean absorbance values from duplicate determinations in a single experiment

3.4.5 Inhibition of telomerase activity in A549 cell lysates

In the following series of experiments, the effect of a series of novel compounds on the telomerase activity in A549 cell lysates was assessed. 34 compounds were screened for telomerase inhibition, of which 19 compounds appeared to inhibit telomerase activity. The inhibitory effects of these compounds were further assessed by determining if the inhibition was a result of non-specific inhibition of the PCR reaction in the assay protocol. A549 cell lysates were incubated with the compounds for 2h as described in section 3.3.1. In order to determine which of the 34 compounds could be possible inhibitors of telomerase activity, the compounds were added to the cell lysates to make a final concentration of 200µM. All 9 water soluble compounds appeared to inhibit telomerase activity (figure 45), whilst only 10 of the 25 DMSO soluble compounds were identified as having inhibitory activity (figure 46). Both experiments were performed twice and figures 45 and 46 show representative results.

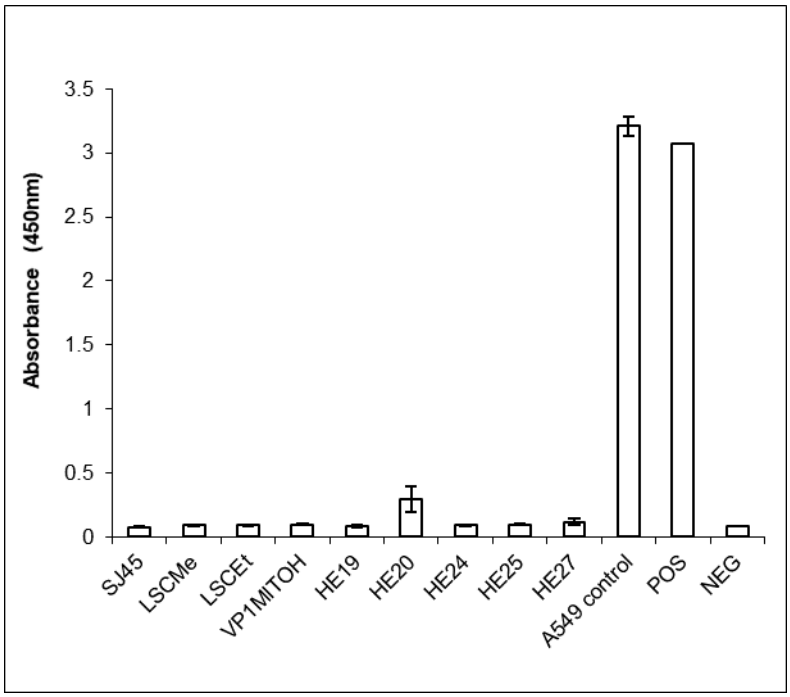


Figure 45 Inhibition of telomerase activity in A549 cell lysates by water soluble compounds (200µM concentration) after 2h exposure. A549 control – A549 cell lysate with water. The results show the mean absorbance values from duplicate determinations in a single experiment.

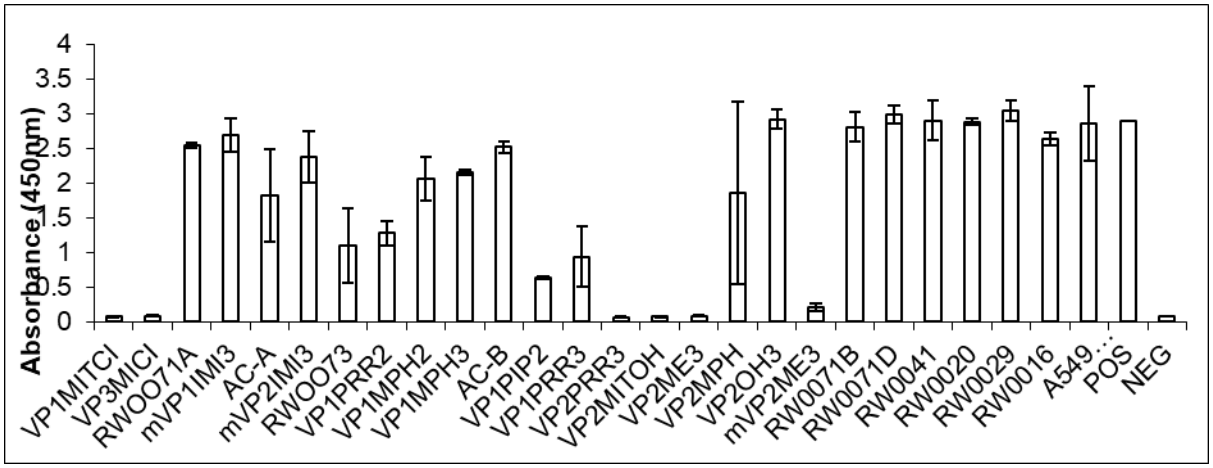


Figure 46 Inhibition of telomerase activity in A549 cell lysates by DMSO soluble compounds (200µM concentration) after 2h exposure. A549 control was A549 cell lysate with 0.1% DMSO. The results show the mean absorbance values from duplicate determinations in a single experiment.

3.4.6 Effect of compounds on telomerase PCR reaction

The first step of the TELOTAGG Telomerase PCR ELISA test is a modification of the original TRAP assay. It has two parts: the first part involves addition of telomeric repeats to biotin labeled synthetic primers by telomerase followed by amplification of these elongation products by PCR. In the second part, the PCR product is denatured and hybridized to a detection probe, this is then immobilized, and the resulting product visualized by ELISA. It was therefore important to eliminate the possibility that the telomerase inhibitory activity of the compounds in the cell free assay was not due to an effect of the compounds on the TRAP assay itself and in particular, the PCR step.

PCR amplification of β actin

To determine if the inhibitory effect of the compounds was as a result of nonspecific inhibition of the PCR reaction, RNA was extracted from A549 cells and reverse transcribed into cDNA. The cDNA was then incubated with the compounds and then subjected to PCR using β actin primers to amplify this house keeping gene. The PCR products were run on an agarose gel. The presence of the β actin PCR product confirmed that:

a) The reverse transcription step was successful
b) The selected compound did not inhibit the PCR reaction

The results for the water soluble compounds are shown in figure 47. The presence of β actin PCR product in lane 2 served as the positive control to which no drug was applied. Lanes 3 and 4 were the negative control lanes using water only. Lanes 6, 7, 9 and 10 were for LSCMe, LSCEt, HE19 and HE20 respectively. The presence of β actin PCR products indicates that these compounds did not inhibit the PCR reaction. These results are representative of three similar experiments. The 8 DMSO soluble

compounds that appeared to inhibit telomerase activity also inhibited the PCR reaction which was indicated by the absence of β actin PCR product on the gel (figures 48).

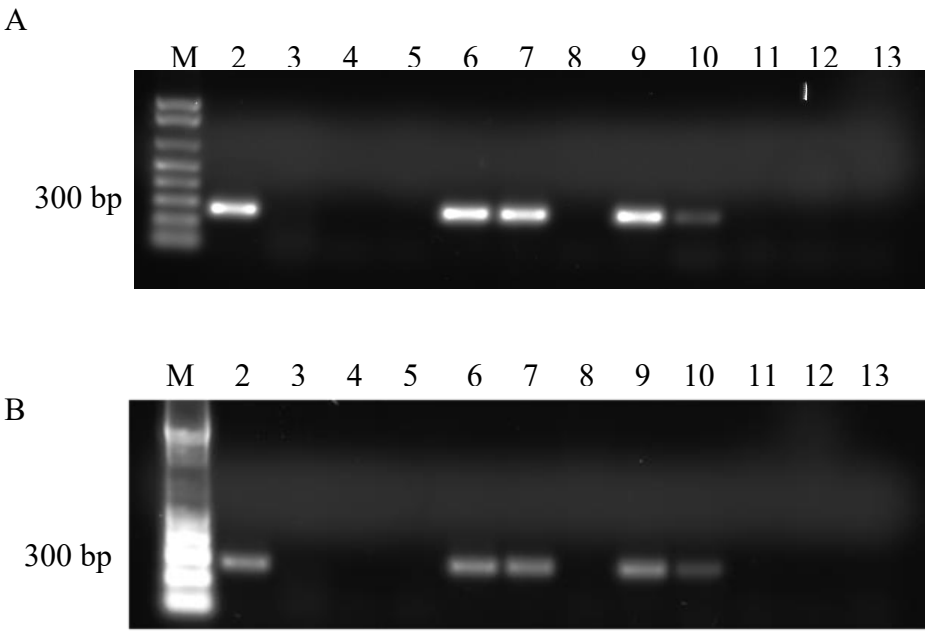


Figure 47 A and B Gel electrophoresis of PCR products showing effect of water soluble compounds on β actin PCR amplification. Lanes: 1 (M – master mix) – DNA ladder, 2 - positive control (β actin PCR product which was not exposed to compounds), 3 - RT negative control, 4 - negative control using water, 5 - SJ45, 6 - LSCEt, 7 - LSCMe, 8 - VP1MIT, 9 - HE19, 10 – HE20, 11 HE24, 12 – HE25, 13 HE27. Lanes 5, 8, 11, 12 and 13 are water soluble compounds which inhibited β actin PCR amplification.

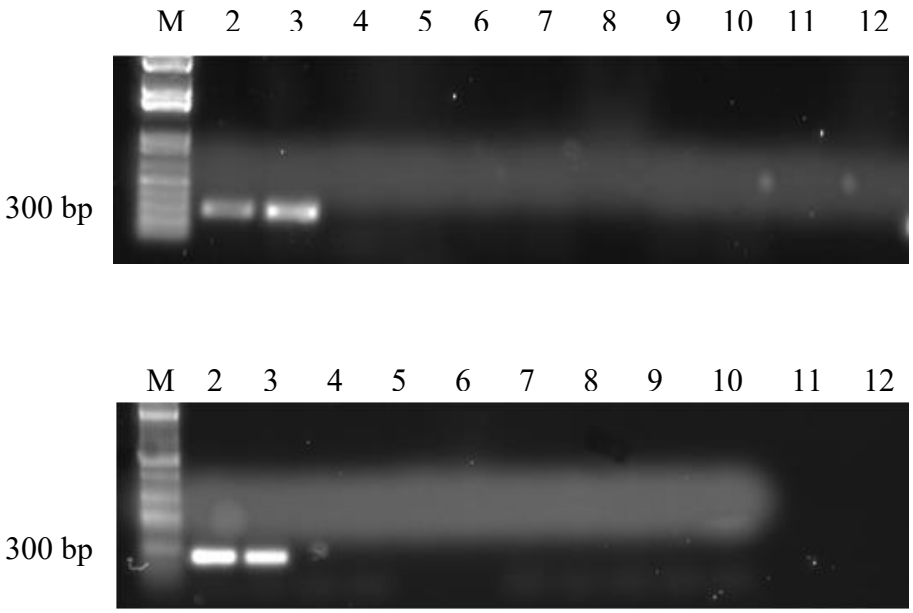


Figure 48 A and B Gel electrophoresis of PCR products showing effect of DMSO soluble compounds on β actin PCR amplification. Lanes: 1 (M – master mix) – DNA ladder, 2 - positive control (β actin PCR product which was not exposed to compounds), 3 - positive control, 4 - RT negative control, 5 – VP1RR2, 6 – VP1PIP2, 7 – VP1PRR3, 8 – VP2MITOH, 9 – VP2ME3, 10 – VP2MPH, 11 - mVP2ME3, 12 – VP2PRR3. Lanes 5 - 12 are DMSO soluble compounds which inhibited β actin PCR amplification.

3.4.7 Effect of selected compounds on telomerase activity

As presented above, HE 19, LSCMe andLSCEt had no inhibitory effect on the PCR reaction (section 4.4.6) and were considered as true inhibitors of the telomerase activity. HE20 partially inhibited Taq. They were therefore selected for further analysis. The 4 compounds were all water soluble and were added to cell lysates to make final concentrations of 200, 100, 50, and 25μM. The results are shown in figures 49 -52 and are representative of 2 independent experiments. There was a 25% decrease in telomerase activity with HE19, HE 20 and LSCMe at 200μM, and 8% decrease with LSCEt. An IC50 value for each compound is shown in table 8.

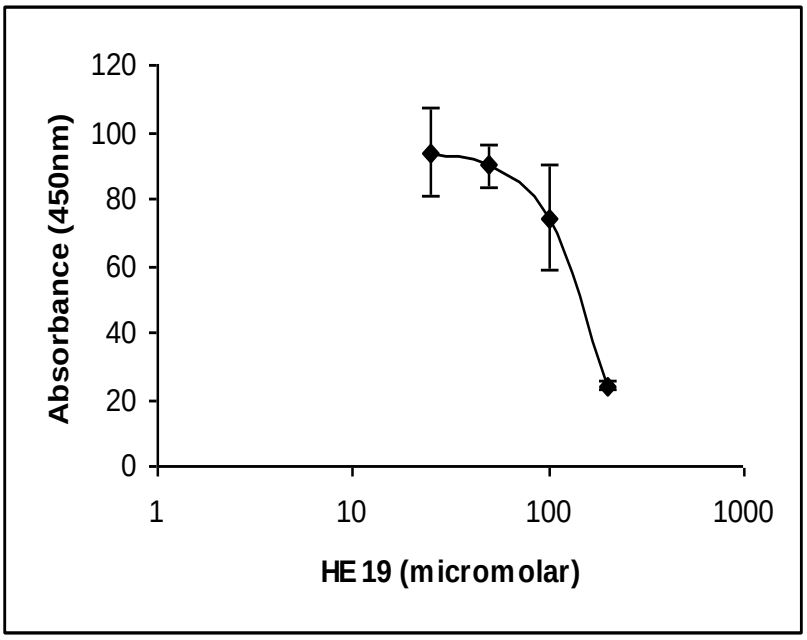


Figure 49 Effect of HE19 on telomerase activity. The results show the absorbance values from duplicate determinations in a single experiment

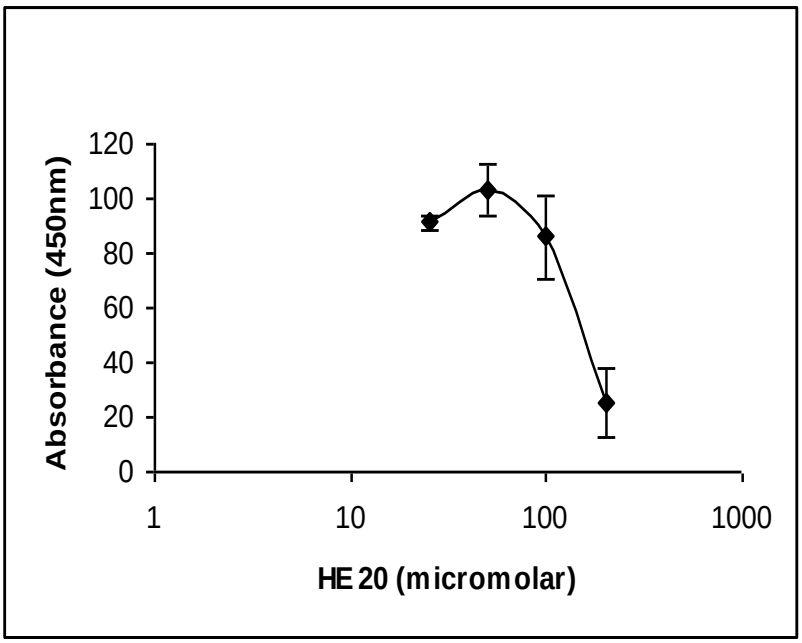


Figure 50 Effect of HE20 on telomerase activity. The results show the absorbance values from duplicate determinations in a single experiment

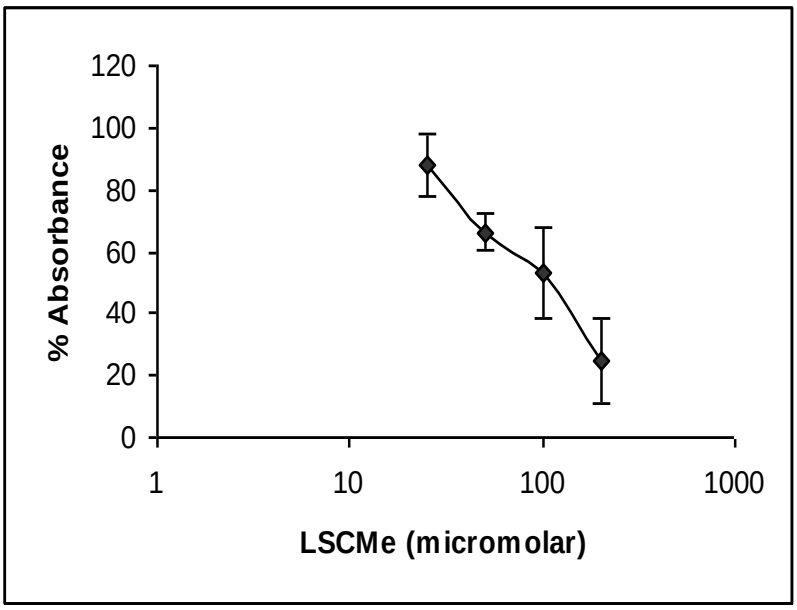


Figure 51 Effect of LSCMe on telomerase activity. The results show the absorbance values from duplicate determinations in a single experiment

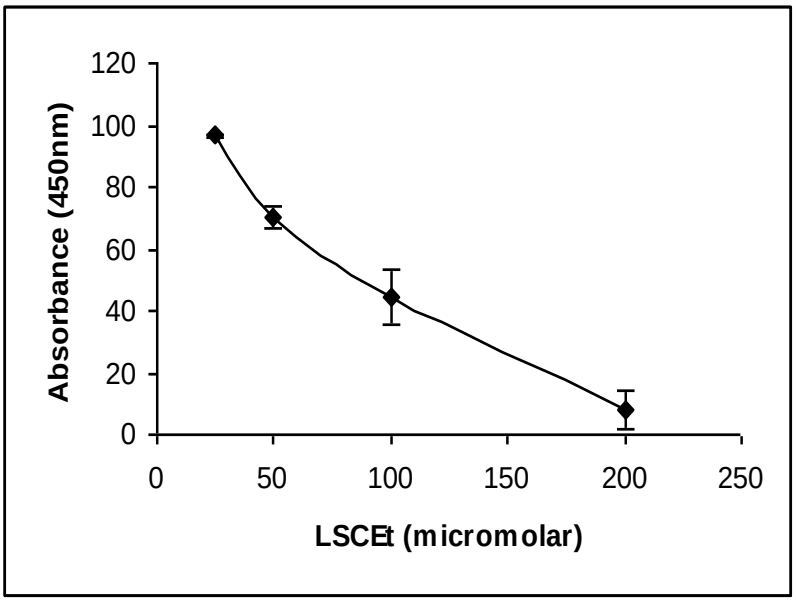


Figure 52 Effect of LSCEt on telomerase activity. The results show the absorbance values from duplicate determinations in a single experiment

Compound	Mean IC_{50} (μM)
HE 19	140
HE20	165
LSCMe	102
LSCEt	89

Table 9 IC_{50} values (concentration at which 50% of telomerase activity is lost) in A549 cell lysates following 2h exposure to the selected compounds (n=2).

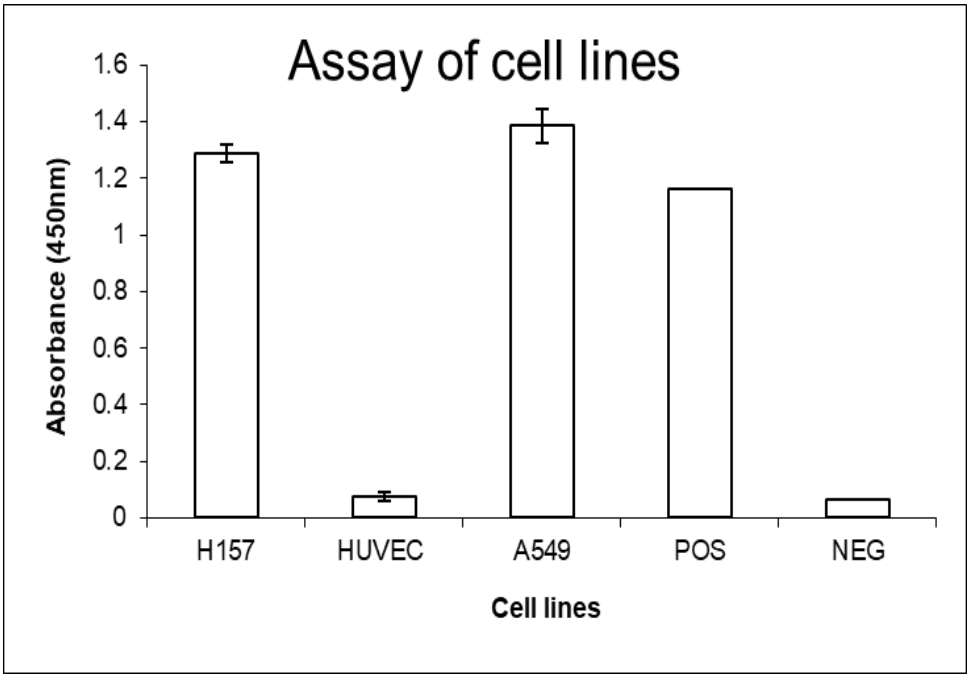
3.5 Cytotoxicity of selected compounds

From the experiments described in section 3.3, four compounds (HE19, HE20, LSCMe, LSCet), were selected for further study (see figure 12 for structures of the compounds). These compounds were able to inhibit telomerase in the cell free assay and did not inhibit the PCR reaction, thus suggesting that they were specifically able to inhibit telomerase activity.

In this section, the relationship between telomerase inhibition and cell cytotoxicity was investigated. Cell lines which expressed different levels of telomerase activity were identified. Two cell lines were selected, were incubated with the selected compounds and cytotoxic effects were assessed using the MTT assay. In order to compare the effect of the selected compounds on telomerase positive and negative cell lines, A549 cells shown to have telomerase activity and HUVEC cells without telomerase activity (figure 53), were also incubated with the selected compounds.

3.5.1 Telomerase activity in cell lines

In order to identify telomerase positive and negative cells, telomerase activity was determined in a range of cell lines. The results are shown in figure 53 (representative of 3 independent experiments). A549, H157, H460, EJ138, HT29, A2780 and HT1080 (all human cancer cell lines), had telomerase activity indicated by the high absorbance values obtained as compared to HUVEC cells and HS27 cells (normal human somatic cells), which showed no telomerase activity.



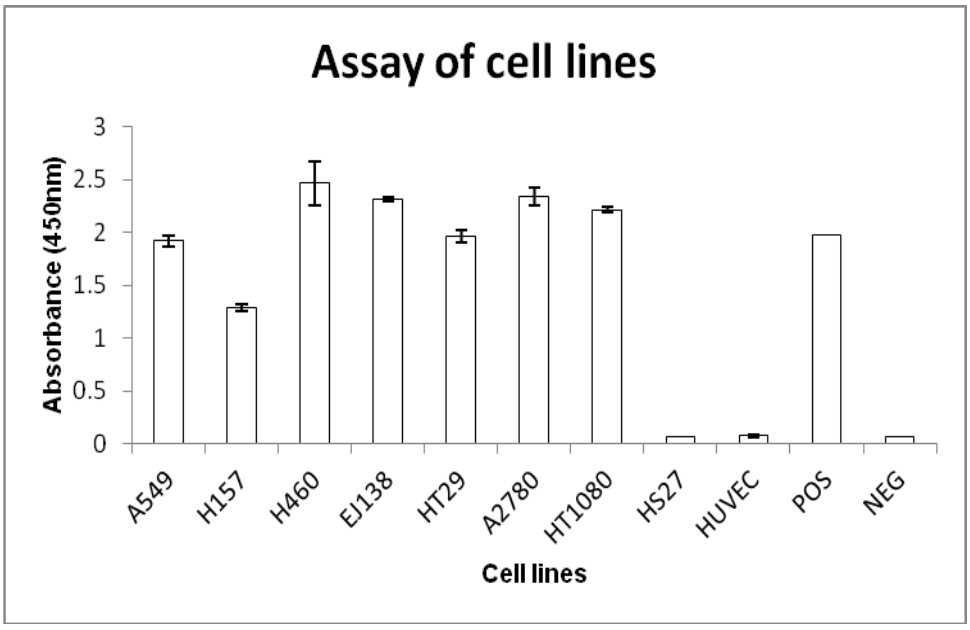


Figure 53 Telomerase activity in telomerase positive and negative cell lines. The results show the mean absorbance values from duplicate determinations in single experiments

3.5.2 The validation of MTT assay

The MTT assay was first validated by confirming that there was a linear relationship between absorbance at 540nm and A549 cell number. The results in figure 54 are representative of 6 independent experiments. The results demonstrate a linear relationship between cell number and absorbance.

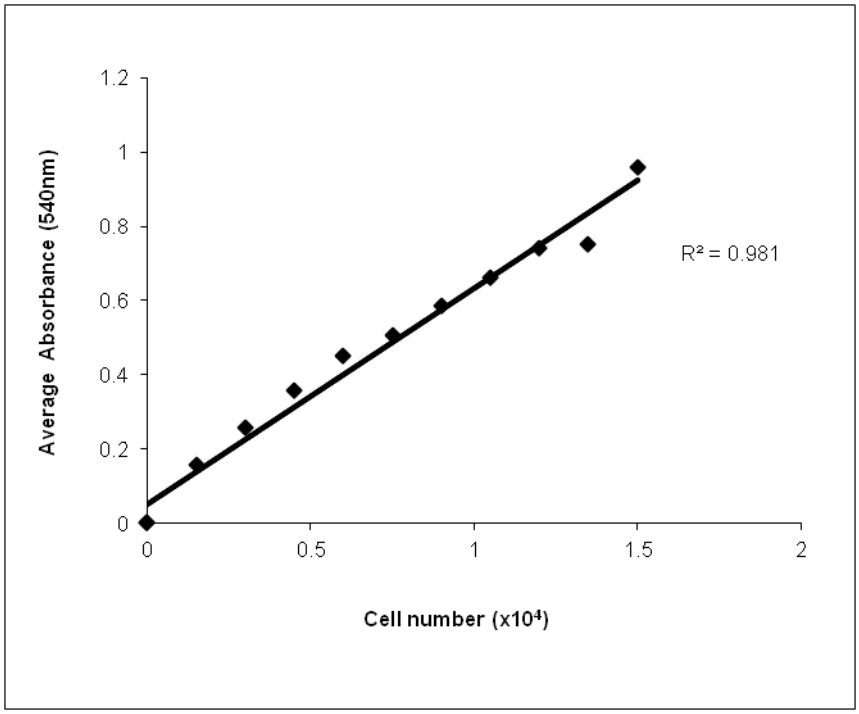


Figure 54 Relationship between A549 cell number and absorbance at 540nm. Results are mean absorbance values from 8 determinations in a single experiment.

3.5.3 Plating of HUVEC cells

1000 cells were seeded into 96 well plates and incubated for 96 hours as was done for the cancer cell lines with resultant poor cell survival. Therefore, in order to determine the optimal number of HUVEC cells that could be used for the chemosensitivity measurements, various concentrations of HUVEC cells in suspension were seeded into 96 well plates and incubated for 96 hours as described

in 3.3.3. The result in figure 55 shows the absorbance values for different concentrations of HUVEC cells. There appeared to be lower absorbance for cell concentration of 40000 (4 X 10^4) suggesting too many cells, and 10000 (1 X 10^4) suggesting too few cells; as compared to 30000 (3 X 10^4) cells and 20000 (2 X 10^4) cells which good absorbance values. 20000 (2 X 10^4) cell concentration was therefore selected as the concentration used for the chemosensitivity measurements.

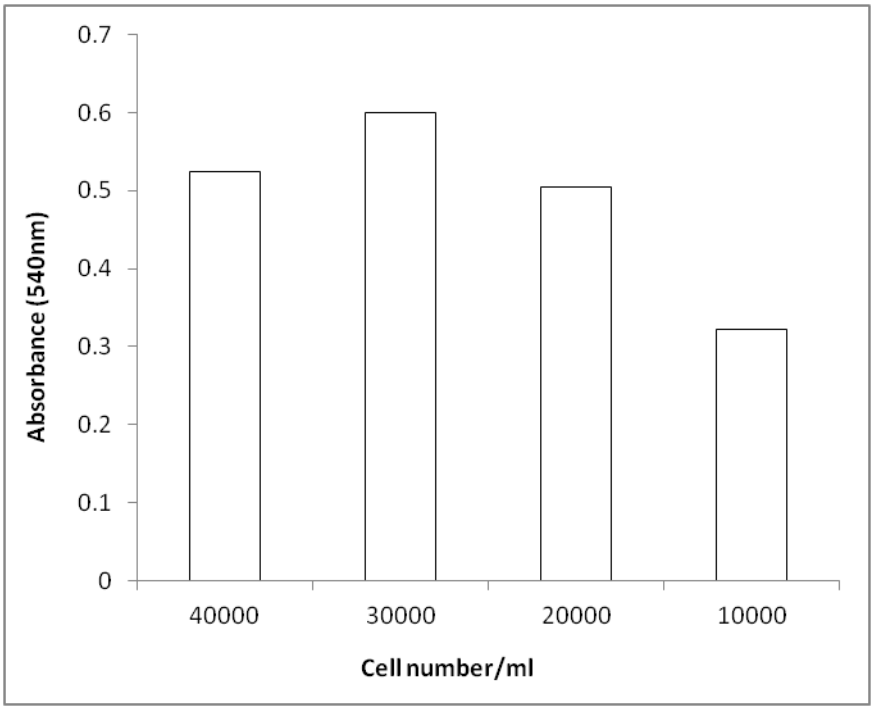


Figure 55 Relationship between HUVEC cell number and absorbance at 540nm. Results are mean absorbance values from 8 determinations in a single experiment

3.5.4 Cytotoxicity of selected compounds *in vitro*

The response of telomerase positive and telomerase negative cell lines (A549 and HUVEC cells) to the 4 selected compounds at 2h and 96 h exposure is presented in figures 56 and 57. There were differences in response to the compounds depending on the duration of exposure. IC_{50} values obtained for both 2h and 96h exposure are presented in tables 10 and 11.

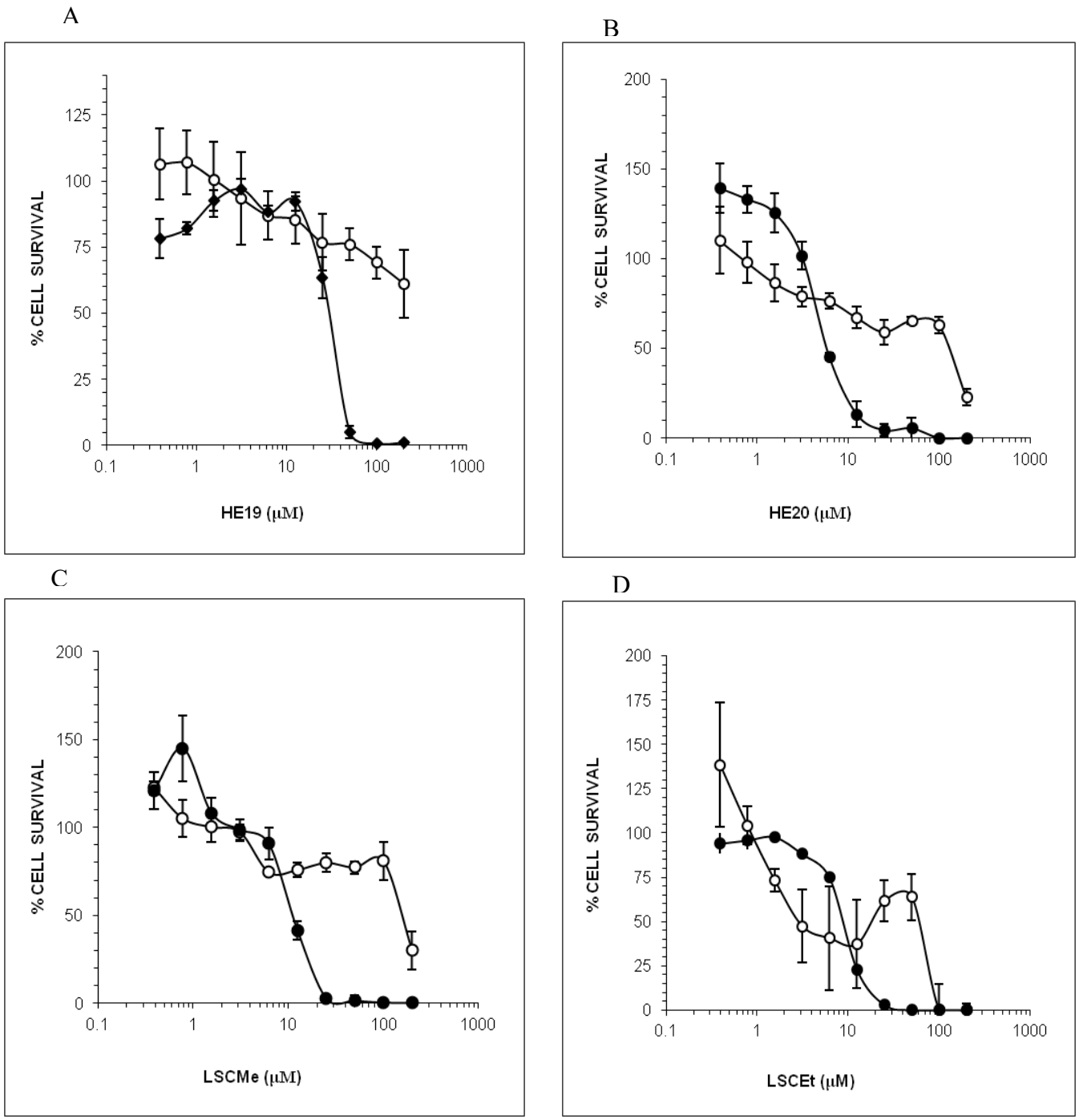


Figure 56 Cytotoxicity of HE19, HE20, LSCMe, and LSCEt on A549 cell line (A, B, C, D respectively). Survival curves were obtained for each compound after 2h (○) and 96h (●) exposure to a range of concentrations. Each value represents the mean ± SEM for three independent experiments. 8 determinations were made in a single experiment.

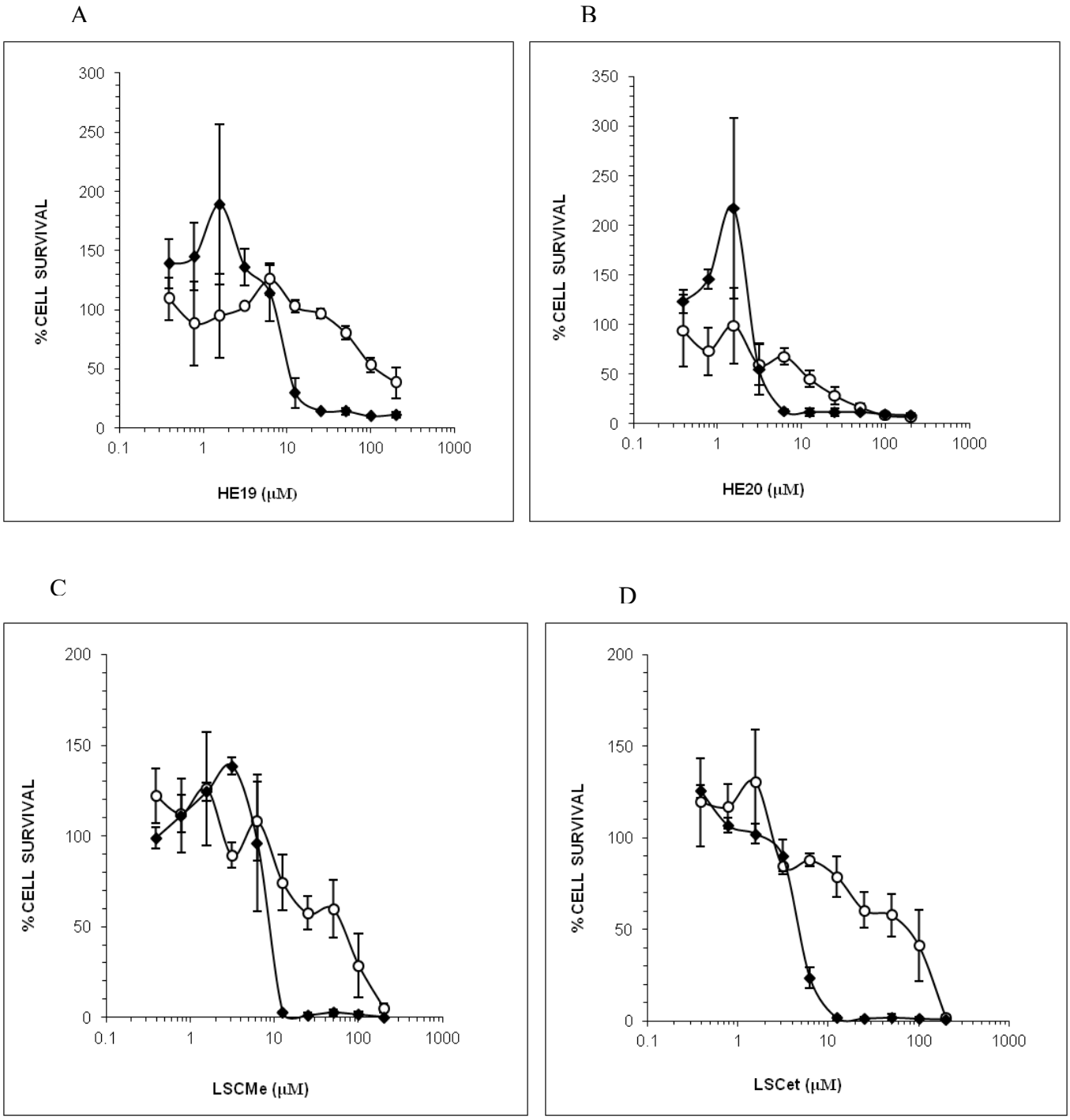


Figure 57 Cytotoxicity of HE19, HE20, LSCMe, and LSCEt on HUVEC cells (A, B, C, D respectively). Survival curves were obtained for each compound after 2h (○) and 96h (●) exposure to a range of concentrations. Each value represents the mean ± SEM for three independent experiments. 8 determinations were made in a single experiment.

Novel compound	Acute cell toxicity (A549 cells)		Cell free assay
	IC_{50} (μM) at 2h	IC_{50} (μM) at 96h	IC_{50} (μM)
HE 19	>200	30	140
HE20	120	6	165
LSCMe	170	11	102
LSCEt	100	10	89

Table 10 Comparison between concentrations of novel compounds needed for acute cell toxicity in A549 cell line and inhibition of telomerase in a cell free assay.

Compound	IC_{50} (μM) at 2h		IC_{50} (μM) at 96h	
	A549	HUVEC	A549	HUVEC
HE 19	120	100	30	10
HE20	120	10	5.8	3
LSCMe	170	60.5	11	8.5
LSCEt	60	40	8.5	4.5

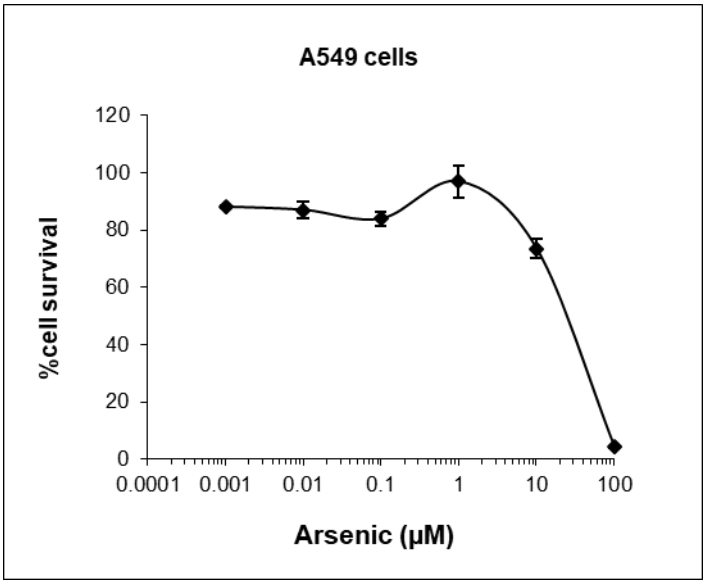
Table 11 IC_{50} values in HUVEC cells and A549 cell line following 2h and 96h exposure to the selected compounds

3.6 Further evaluation of selected compounds

The difference in telomerase activity and telomere dynamics between cancer and normal cells and also the telomere/telomerase complex offers multiple possibilities for inhibition. The selected compounds were previously shown to inhibit telomerase activity (4.4.5). In the following series of experiments, the effects of the selected compounds on telomerase activity were evaluated further. Here, the aim was to determine if the impairment of telomerase function and growth inhibition of the tumour cells would lead to induction of senescence. The β galactosidase assay was used to determine if growth inhibition led to the occurrence of senescent cells. Cell lines selected were the PC3 and A549 cell lines which are both known telomerase positive cell lines (Figure 14; Sommerfield *et* al., 1996). Arsenic was selected as the control compound to exhibit senescence (Zang et al. 1999; Chou et al. 2001).

3.6.1 β galactosidase assay

It has been shown that interference with telomere architecture and maintenance can initiate senescence (Karlseder et al 2002, Leonetti et al 2004). Gowan et al in 2004 showed that a pentacyclic acridine (RHPS4) was found to be a potent telomerase inhibitor in the TRAP assay and to cause irreversible cessation of growth after long term culture at non-cytotoxic concentrations in cancer cells. In order to determine if the selected compounds were able to cause senescence in nontoxic doses, the following experiments were performed. First of all, A549 cells and PC3 cells were incubated with arsenic and acute cytotoxic effects were assessed using the MTT assay as shown in figure 58. Sub-toxic doses (concentration of compound at which 90% of cells were viable) were then determined (table 12).



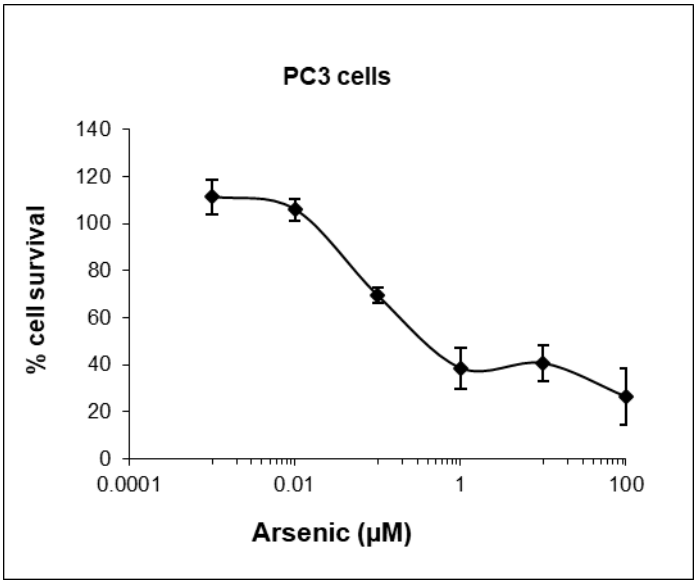


Figure 58 Cytotoxicity of Arsenic on A549 and PC3 cell lines. Survival curves were obtained after 96h exposure to a range of concentrations. Each value represents the mean ± SEM for three independent experiments. 8 determinations were made in a single experiment.

Cell line	Arsenic (sub-toxic dose µM)
PC3	0.02
A549	0.01

Table 12 Sub-toxic values (concentration of compound at which 90% of cells were viable) in PC3 and A549 cells following 96h exposure to arsenic

PC3 and A549 cells were then incubated with the selected compounds, the cytotoxic effects were assessed, and sub toxic doses were determined (figures 56 and 59; table 13).

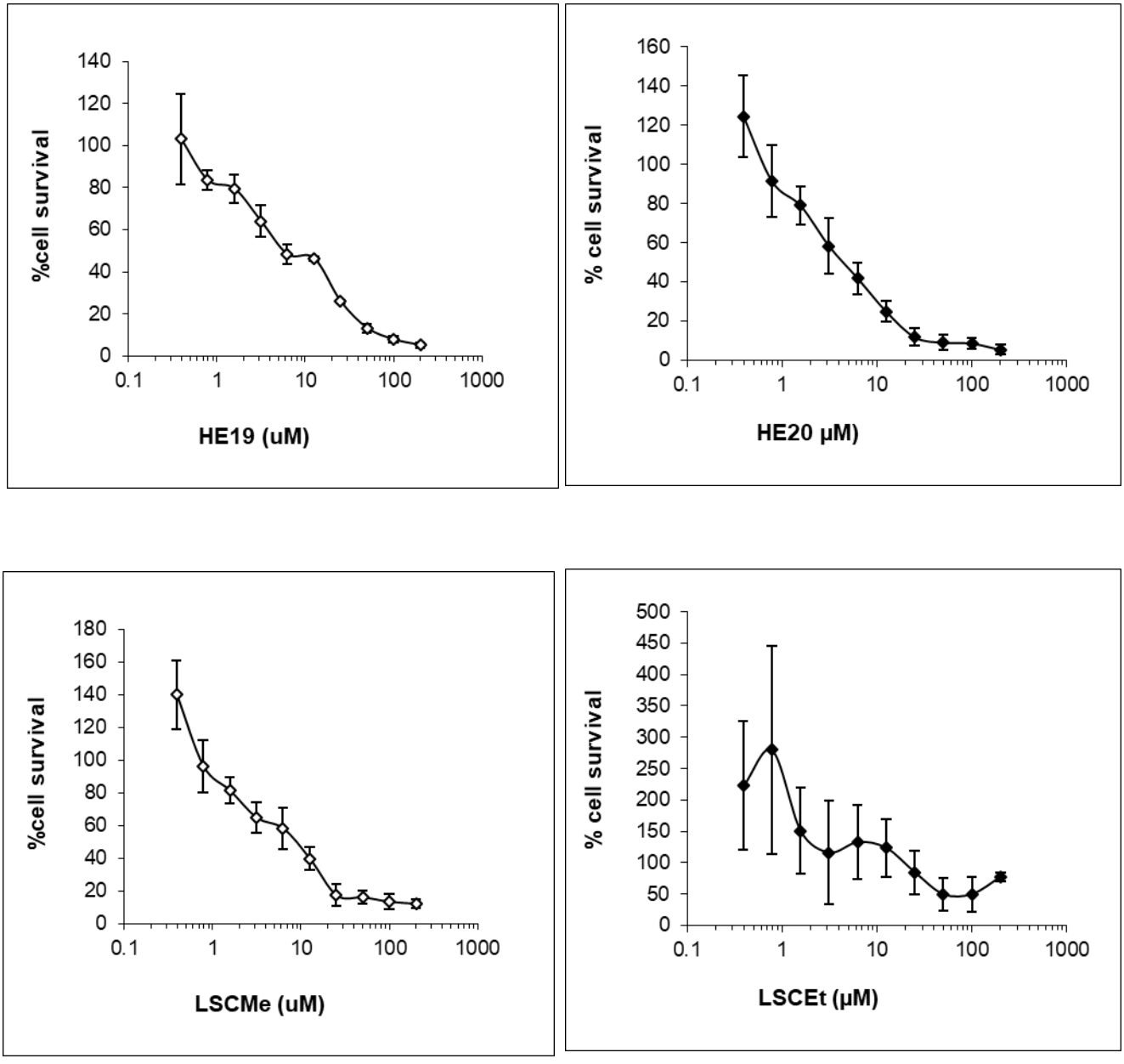


Figure 59 Cytotoxicity of selected compounds on PC3 cell line. Survival curves were obtained after 96h exposure to a range of concentrations. Each value represents the mean ± SEM for three independent experiments. 8 determinations were made in a single experiment.

Compounds (sub-toxic doses μM)	PC3	A549
HE19	0.75	3
HE 20	0.6	3
LSCMe	1.5	3
LSCEt	1.5	1.5

Table 13 Sub toxic values in PC3 and A549 cells following 96h exposure to the selected compounds

In order to perform the β galactosidase study, the cells were incubated with arsenic and the compounds in 6 well plates at 37^0C as previously described (3.3.5). Controls had no compounds added. PC3 cells and A549 cells were exposed to sub-toxic doses of arsenic and sub-toxic doses of the selected compounds for 5, 10 and 15 days after which the plates were examined for the presence of senescent cells. There was absence of senescent cells after 5, 10 and 15 days in both the plates exposed to arsenic as well as to the plates exposed to the selected compounds. This implied that the mechanism of cell toxicity of the compounds was not via the induction of cellular senescence.

4 DISCUSSION

Cancer is a major disease burden worldwide and deaths from cancer in the world are projected to continue rising. It is a major cause of mortality and morbidity in the UK with more than 1 in 3 people in the UK developing some form of cancer in their lifetime. Cancer is therefore a condition with major socioeconomic impact requiring great efforts to improve current strategies for detection and treatment.
HNSCC is a complex disease and despite therapeutic advances in its treatment, the overall 5 year survival from the disease has remained unchanged over the last 30 years due to diagnostic and treatment challenges. Two issues that would positively affect the outcome in HNSCC patients are (i) early diagnosis and (ii) targeted treatment.

The thesis submitted here had 2 general aims: firstly, to investigate the feasibility of detection of telomerase activity in saliva of patients diagnosed with upper aero-digestive tract squamous cell carcinoma using a nonradioactive telomerase PCR-ELISA method. The aim was to develop a simple technique for detection in both primary and recurrent disease. Secondly, to screen a range of novel compounds with a view to identifying effective and novel telomerase inhibitors.

4.1 Detection of telomerase activity in saliva

The first part of this study was to determine the presence of telomerase activity in saliva. Salivary diagnostics using salivary biomarkers specifically for early cancer detection has attracted research interest especially for cancers occurring in the oral cavity and oropharynx. Sankaranarayanan et al (2013) showed that oral cancer screening reduced oral cancer mortality by more than 80 percent in tobacco and/or

alcohol users. However, Brocklehurst et al (2010) had previously shown that this method of screening which included visual inspection followed by tissue biopsy had only 64 percent sensitivity for oral cancer and 31 percent specificity for oral dysplasia or cancer. The detection of telomerase activity in saliva of head and neck squamous cell cancer patients could be used in conjunction with histological analysis to improve screening, diagnosis/staging and treatment. It would be useful as a molecular marker, helping to reduce the morbidity and mortality currently associated with the disease and improve the prognosis in patients. Therefore, much research effort has been dedicated to investigating potential salivary biomarkers. Salivary biomarkers represent a promising non-invasive approach for oral cancer detection, and an area of strong research interest. Of the many oral cancer biomarkers studied, only a few are being translated to clinical practice. An example of this is CD44, a cell surface transmembrane glycoprotein which has been found to be a key tumor initiation marker, over-expressed in the earliest stages of carcinogenesis and has been shown to be an effective tumor marker. Together with total protein, it is detectable in body fluids and can be measured with simple, inexpensive assays (Hirvikoski et al 1999; Dasari et al 2014; Franzmann et al 2012).

Telomerase activation has been shown to be an early event in the progression of HNSCC (Mao et al 1996). Telomerase activity is commonly determined using the TRAP assay which allows semiquantitative and quantitative analysis. In the standard TRAP assay, originally described by Kim et al (1994), PCR products were detected in polyacrylamide gels using a radioactive label which was introduced using a radioactively labeled primer. The drawback of this technique was the radioactivity. In this study, the TRAP assay was the preferred method of determining telomerase activity, using the telomerase PCR ELISA kit. This provided a way to detect

telomerase activity using a non-radioactive technique. The telomerase PCR ELISA kit has been used by several investigators to detect telomerase in some body fluids such as urine (Melissourgos et al. 2003), pleural and ascitic fluid (Yamamoto and Hirakawa 2004). Oral rinses of HNSCC patients have also been examined previously using radioactive based methods of the TRAP assay (Califano et al 1996; Mutiranga et al 1996; Sumida et al 1998). Telomerase activity was detected by these investigators, but with a low positive rate. Califano et al in 1996 detected telomerase activity in 14 of 44 (32%) oral rinses from patients with HNSCC. They used a modification of the method first described by Kim et al (1994), and the PCR products were amplified using a radioactive method. This low positive rate was thought to be due to a number of factors including the presence of RNase in saliva or storage of the saliva samples for prolonged periods at room temperature, both of which could degrade the telomerase enzyme activity. A low percentage of cells in the samples, of which few were cancer cells, were also thought to be a possible reason for low positivity.

The first aim of this study was to attempt to use a simplified, nonradioactive based technique to detect telomerase activity in saliva of HNSCC patients who had a histological diagnosis of the condition. This was beneficial as it avoided the use of radioactive substances. To my knowledge; no other investigators at the time had attempted to do this using the non radioactive technique.

First of all, the Haemotoxylin and Eosin stain was used to demonstrate the presence of epithelial cells in saliva (figure 18). The viability of these epithelial cells was then determined by trypan blue exclusion (figures 19). This demonstrated the presence of epithelial cells in the saliva smear which were then shown to be viable.

Determination of the exact number of epithelial cells present in the saliva samples could not be carried out reliably due to the significant amount of debris in the samples. This was despite ensuring saliva was collected at least 2 h or more after consumption of food or drink.

As previously mentioned, it has been shown that saliva contains endogenous RNA (Li et al 2004) as well as ribonucleases (Eichel et al 1964; Bardon and Sugar 2004), but it is unclear how these coexist. These RNases could potentially degrade the RNA subunit of the telomerase enzyme. In this study, it was shown that the telomerase enzyme activity was preserved despite the presumed presence of endogenous RNA in saliva. A549 cell pellets (produced from 2 x 10^5 cells, stored at -80^0C) were added to the saliva samples obtained by asking volunteers to gargle, in order to determine if the telomerase enzyme would be active in the presence of saliva and under the storage conditions of -80^0C. The saliva samples were immediately tested for telomerase activity after addition of the cells and results showed that added telomerase could be detected in the saliva samples indicating that telomerase activity was preserved in saliva. The telomerase activity also did not appear to be affected by the storage condition of the cell pellet at -80^0C. In addition, as seen in figure 20, neither saliva nor the solutions used for collection affected the telomerase activity.

As saliva is a viscous fluid, it was noted that centrifugation of the saliva samples without further addition of either Hartmann's or saline solution, produced a poor pellet. Several methods of collecting saliva samples were therefore tried in healthy controls to improve the yield of cells and formation of pellets after centrifugation. Comparing the methods of using 3 ml of solution to gargle followed by addition to the sample of a further 15 ml of solution and using 10ml of solution to gargle (without

further addition of solution), there was no apparent difference in the telomerase activity obtained (figure 21). In both cases, $2x10^5$ A549 cells were added to the samples prior to centrifugation. Centrifugation at 1000*g* and 1500*g* was compared, and it appeared that a better pellet was obtained using 1500*g*.

The degree of sensitivity of the telomerase PCR ELISA kit was determined by looking at the effect of serially diluting cells before lysates were prepared and serially diluting the cell lysates themselves. When the cells were serially diluted, there was a linear relationship between cell concentration and absorbance values obtained in the assay when cell number was above 1000 cells per ml of suspension. Below 1000 cells/ml, there was a sharp drop in the absorbance value, and even though the kit detected telomerase activity at cell counts as low as10 cells/ml, there was inconsistency in the absorbance values detected below 1000 cells/ml (figure 15). The cell lysates were also serially diluted in order to determine degree of sensitivity of the kit, by determining the lowest protein concentration that would produce a high absorbance (indicating positive telomerase activity). The value obtained appeared to be in the range of 0.02µg/µl to 0.8µg/µl (corresponding to a total amount of protein in the assay of 40ng to 1.6µg) as shown in figure 16. This was produced from 5000 – 200000 cells.

In this study, saliva samples in form of oral rinses were collected from a total of 28 subjects. 14 patients with oral cancer and 14 control subjects. Patients with oral cancer were selected from the head and neck cancer patients in order to provide a best possible chance of collecting representative tumour cells. Samples of saliva collected from the patients failed to show distinct telomerase activity (figure 24). As shown previously, telomerase activity could be detected using volumes of lysates

which contained a minimum of 40ng of protein (figure 16). In the cell lysates obtained from the patient samples, the amount of protein which was used in the PCR reaction was in the range of 50 ng to 20µg (figure 22). These results indicate that there should have been enough cells to produce lysates in the saliva sample which would produce detectable telomerase activity, if the cells were expressing the enzyme. Figure 19 showed that viable cells were demonstrated in the saliva samples and the telomerase PCR ELISA kit can detect cell counts as few as 10 cells/ml (figure 15). The absence of telomerase activity in patient samples could therefore have been as a result of low numbers of cancer cells in the saliva samples which may have been below the threshold needed for detection or there were no telomerase positive cells shed into the patient samples. Califano et al (1996) noted that the destruction of the telomerase enzyme by RNases could have been a factor responsible for poor detection. In this study we were able to demonstrate that exogenous telomerase enzyme activity was maintained in the presence of saliva. Another factor for failure to detect telomerase activity in the patient samples could be the collection and storage conditions of the samples. The samples were collected and immediately placed on dry ice, then centrifuged within 10 min after which they were frozen and stored at -80^0C until use. There is a possibility that enzyme activity was lost within this time period. However, A549 cells used in a series of experiments (figures 14) were obtained from cell pellets stored at -80^0C which did not affect the telomerase activity.

Ohyashiki et al (1997) were able to specifically identify telomerase positive cells in range of cell lines using *in situ* PCR. In the future, this method might be a possible way of determining if the cells seen on the saliva smears were actually cancer cells and help as a further diagnostic aid.

4.2 Detection of hTERT protein

Human telomerase consists of two major components: human telomerase RNA (hTR) which acts as a template for reverse transcription, and human telomerase reverse transcriptase (hTERT), the catalytic subunit which has reverse transcriptase activity. hTERT is believed to be a critical step in the production of telomerase activity. The expression of hTERT subunit has been shown to correlate well with telomerase activity and hTERT gene expression is generally repressed in normal human cells and up regulated in tumour cells (Meyerson et al 1997, Nakayama et al 1998, Ritz et al 2005). Hiyama et al in 2001 were able to detect the presence of hTERT protein at the cellular level in paraffin embedded tissues which had previously been reported to have telomerase activity. They were able to detect hTERT protein using immunohistochemistry in cultured cells and tissue sections obtained from a variety of tumour tissues such as breast, lung, stomach and colon. They showed that there was little background nonspecific staining of cell types that were believed to be telomerase negative. H157 is a human oral squamous cell carcinoma cell line and telomerase activity was shown to be present in this cell line using TRAP assay (figure 53). HUVEC cells (which are normal human cells), were shown not to possess telomerase activity (figure 53).

As there was failure to detect telomerase activity in the saliva of the selected patients, a further study was done to determine the presence of telomerase in the selected oral cancer patients from whom saliva samples were obtained. Here, we aimed to determine the presence of telomerase hTERT protein in formalin fixed, paraffin embedded tissue samples of the patients using immunohistochemistry.

First of all, the presence of hTERT protein expression was determined in H157 cells using immunocytochemistry. Three different dilutions of the primary antibody (1:200, 1:100 and 1:50) were used to determine the dilution that would produce the best intensity of immunostaining. The presence of brown staining was indicative of hTERT expression. The most intense brown staining was obtained using the 1:50 primary antibody dilution (figure 25). 1:50 dilution of the primary antibody was therefore selected as the primary antibody concentration used. There was no staining observed in the HUVEC cells or negative controls (which had no antibody) (figures 26 and 27). This finding is indicative of the correlation between the presence of telomerase activity and the expression of hTERT.

Next, the presence of hTERT protein in patient tissue samples was assessed using immunohistochemistry. The methodology was developed by using A549 xenografts derived from subcutaneously grown tumours in NCR/Nu mice. First of all, two different antigen retrieval methods were compared: one using the microwave and another with the oven. Three different dilutions of the primary antibody were used (1:200, 1:100 and 1:50), the negative control was without antibody. In the experiments performed using the microwave technique, different time periods of microwave heating were performed - (20 min, 10 min and no microwave heating). In order to determine the best method of antigen retrieval, both the microwave method and the oven method of antigen retrieval were performed. The microwave technique of antigen retrieval involving heating the slides for 20 minutes was selected for further experiments as there was no difference in the intensity of staining using the different methods of antigen retrieval (figures 28 – 34). 1:50 dilution of the primary antibody (figure 30A) was selected as the antibody dilution used for further studies as this produced the best intensity of brown staining. Paraffin embedded tissue

samples were obtained for 7 out of the 14 patients and in all cases, there was brown staining indicative of hTERT protein expression.

In this study, using the TRAP assay, saliva samples from all 14 patients did not show telomerase activity; however, it was possible to detect hTERT protein expression in 7 of the 14 patient tissue samples. Despite the failure to detect telomerase activity in the saliva samples of selected patients, the detection of hTERT protein expression in some patients indicates that there was likely to be telomerase activity present in the patient's tumour cells, but we were unable to detect it in saliva for reasons stated above. Work on salivary biomarkers for oral cancer detection has been extensively reviewed by Cheng et al in 2014 and emerging issues / challenges preventing the use of reliable salivary biomarkers in the clinical setting were identified. These issues include a lack of standardization for saliva sample collection, processing, and storage; and wide variability in the levels of potential oral cancer salivary biomarkers in both non-cancerous individuals and oral cancer patients. These issues and challenges are likely applicable in this study.

4.3 Telomerase inhibition

The management of malignant disease remains one of the most challenging areas of modern medicine, and despite advances in the use and development of conventional cytotoxic agents, the cure rate remains disappointing in most patients with advanced disease of common solid tumours (Keith et al, 2002). This poses a problem especially in head and neck cancer patients in whom a significant proportion present with locally advanced and unresectable disease. Research into the mechanisms of cancer pathogenesis as discussed previously has resulted in the introduction of

mechanism based, targeted therapies to treat cancers which can be categorized according to their effects on one or more of the hallmark capabilities (figure 4; Hanahan and Weinberg 2011). Targeting the hall mark ‘enabling replicative mortality’ by looking at possible telomerase inhibitors has been a focus of this thesis.

Telomerase activity is usually repressed in human somatic tissues and highly active in most tumours (section 1.7). Telomerase activity has been validated as an anticancer drug target in studies that reported that the over expression of dominant – negative hTERT mutants resulted in telomerase inhibition with subsequent telomere shortening and eventually senescence or crises (Hahn et al, 1999; Zhang et al, 1999). Telomerase can therefore be considered an attractive target for cancer therapy as cancer cells would be targeted whereas, the remaining healthy tissue would be largely unaffected. In addition to this, there is also a possibility that telomerase inhibitors may be employed in combination with established cytotoxic therapy to aid their effectiveness (Baudino et al. 2015). Different approaches to the inhibition of telomerase activity have been under development for more than two decades. Various approaches to the inhibition of telomerase expression has been reviewed by Jarstfer et al (2013). Mechanisms by which this is done include directly targeting the telomerase (hTERT or hTR) or targeting the telomere, targeted gene therapy and telomerase-based immunotherapy of cancer (figure 10; table 2). Examples of previously investigated compounds include small molecules such as AZT (3’-azido-2’, 3’-dideoxythymidine) which are nucleoside analogues with reverse transcriptase inhibitory effect and therefore would potentially inhibit hTERT (Strahl and Blackburn 1996; Melana et al 1998). These compounds lacked selectivity for telomerase, and this therefore limited this approach.

BIBR1532 is a nonnucleoside inhibitor of telomerase which has been extensively studied *in vitro and in vivo* (Damm et al 2001; Pascolo et al 2002). BIBR1532 demonstrated potent inhibition of telomerase in cell free assays. The draw back with this group of compounds was that cellular effects required an extensive period of exposure to the compounds (~100 days), to allow the telomeres to shorten to critical lengths.

Another strategy at targeting telomerase has been through various antisense-based molecules of which the most advanced is GRN163L. GRN163L, also called Imetelstat, has been one of the most widely developed direct inhibitors of telomerase. It acts as a direct telomerase inhibitor by antagonistically binding to the RNA template of telomerase (hTR). GRN163L has been shown to inhibit telomerase activity with resulting senescence and apoptosis after progressive telomere shortening in various cell lines (Asai et al. 2003; Gellert et al. 2006; Chiappori et al. 2015). Imetelstat has been undergoing clinical trials for several years. Recent clinical development of Imetelstat include studies on patients with myelofibrosis (IMbark™ study), and on patients with myelodysplastic syndrome, called (IMerge™ study). These studies are currently recruiting targeted patients at various centers in the USA, Europe and Asia.

The telomerase inhibitors such as GRN163L which target telomerase components act by causing progressive telomere shortening and induction of cellular senescence after a lag phase. The possible drawback of this is that it would take a significant amount of time for the action of the drug to become apparent, but if used in conjunction with other agents, the effect might be beneficial. For this reason, telomerase inhibitors are now being tested in combination with conventional cancer

therapy and in maintenance clinical trials aimed at reducing recurrence after surgery, chemotherapy or radiation therapy (Baudino et al. 2015).

Several compounds have been developed to target the telomere and some of these exploit the ability of G – rich sequences of DNA like telomeres to fold into 4 stranded (quadruplex) intramolecular structures. The novel compounds used in this study can be described as G – quadruplex stabilizers. The stabilization of G-quadruplexes can prevent telomerase from accessing and elongating the telomere. As mentioned previously, telomeric DNA is capable of folding into four-stranded guanine (G4) quadruplex structures and in order for telomeric DNA extension to occur, telomerase must hybridize to its DNA primer which it is unable to do in the presence of G - quadruplex formation. This offers a potential means to selectively target telomeric DNA rather than other regions of the genome (Zahler et al 1991).

Increasing evidence has shown that most G- quadruplex ligands confer their biological activity through interaction with telomeric G-rich single strand overhang thereby affecting telomere capping state and leading to relatively rapid senescence and or apoptosis (Karlseder et al 2002; Salvati et al. 2007). This is compared to direct telomerase inhibitors (such as GRN163L and BIBR1532), which require a lag period (during which time there is loss of the telomere), before the effect of drug becomes apparent. The therapeutic potential of G quadruplexes ligands has resulted in a rapidly increasing number of studies in which small molecule ligands have been used to act as G quadruplex stabilizers (Shalaby et al. 2013). However, cellular and in vivo data are only available for a small number of these compounds (Neidle. 2010). A range of G – quadruplex interactive compounds has been developed over the years which have demonstrated telomerase inhibition. Disubstituted anthraquinones (Sun et al 1997) were the first small molecule G – quadruplex

telomerase inhibitors described. One of the most studied small molecule G-quadruplex stabilizer is Telomestatin. Telomestatin is a natural product which has the ability to inhibit telomerase activity at nanomolar concentrations. It was first isolated from the bacteria *Streptomyces Anulatus* by Shin-ya and co-workers in 2001 (Shin-ya et al. 2001). The IC50 value of telomestatin was calculated based on modified TRAP assay methods. Since telomestatin interferes slightly with PCR reaction, the IC50 values ranged from 58 to 600 nM with various primers in variants of TRAP assay method (Reed et al. 2008). Telomestatin was found to impair glioma stem cell survival and growth through the disruption of telomeric G quadruplex (Miyazaki et al. 2012). So far, no study of these compounds has as yet progressed to clinical evaluation.

Other G - quadruplex inhibitors described include cationic porphyrins such as TMPyP4 (Fujimori et al. 2011; Mikami-Terso et al 2009), trisubstituted acridines such as BRACO19 (Burger et al. 2005), and pentacyclic acridines such as RHPS4 (Gowan et al 2001; Salvati et al. 2007). One of the draw backs of early G quadruplex inhibitors was the relative lack of selectivity for 4 (quadruplex) versus 2 (duplex) stranded DNA which resulted in similar potencies for inhibiting telomerase *in vitro* and for producing non-specific acute cell toxicity. Quarfloxin is a G quadruplex inhibitor that reached clinical trials. It reached phase 2 clinical trials for the treatment of neuroendocrine tumours and chronic lymphoid leukaemia (Shalaby et al. 2013).

The second aim of this study was to identify an effective inhibitor of the telomere/telomerase complex by screening a selected group of compounds. The compounds used in this study were designed to target telomeric DNA quadruplexes, stabilize them, making them inaccessible to telomerase and therefore indirectly result in inhibition of telomerase activity. The anti-telomerase and cytotoxic activity of the compounds was investigated.

4.3.1 Telomerase inhibition by selected compounds in cell free assay

First of all, the compounds were screened in a cell free assay to determine which compounds would inhibit telomerase activity. The effect of the compounds on telomerase activity was assessed *in vitro* using lysates of A549 cell line – a telomerase positive cell line (figures 14). This was assessed using the TRAP assay within the telomerase PCR ELISA kit. The methodology was developed by using berberine chloride – a known moderate telomerase inhibitor as a control. First of all, the stability of the enzyme at 37^0C was assessed over a varying time period (30min to 4 h), in order to select the best time period over which to expose the lysate to drugs. The enzyme appeared stable from 30min to 2h; but from 3h to 4 h there was progressive reduction in the activity of telomerase at 37^0C. 2h was therefore selected as the time period for exposure to drugs (figure 39).

As DMSO was to be used as solvent in some of the selected compounds, the effect of DMSO on the TRAP assay was assessed by exposure of the lysates to different concentration of DMSO (5% to 0.25%). There appeared to be no effect of DMSO on the absorbance values obtained in the assay and therefore telomerase activity (figure 40).

The effect of established chemotherapeutic agents such as doxorubucin, 5 fluorouracil and cisplatin were also assessed (figure 41). Doxorubicin appeared to inhibit the telomerase activity, consistent with the previous finding of Zhang et al (2002). In contrast, Cisplatin showed no inhibition of telomerase activity although it has been reported as a telomerase inhibitor by Burger et al (1997).

Cell lysates derived from $2x10^5$ cells were initially exposed to different concentrations of berberine chloride without incubation of the lysate with berberine chloride. The results showed lack of inhibition of the enzyme, indicating that the drug was not effective without incubation. The lysates were then incubated in 200µM of berberine chloride over different time periods (figure 43) and this showed 2h to be the time at which maximum inhibition occurred. The lysates were again incubated in a range of concentrations of berberine chloride for a 2h period at 37^0C and this resulted in 50% loss of telomerase activity when incubated in 200µM concentration of berberine chloride (figure 44). 200µM was therefore selected as the concentration of the selected compounds for use. As berberine chloride was used to develop the assay protocol, it was not investigated further. The cell lysates were exposed to 200µM concentration of the novel compounds over 2h at 37^0C. 34 compounds were screened - 9 water soluble compounds and 25 DMSO soluble compounds. Of these, all 9 water soluble compounds and 10 of the 25 DMSO soluble compounds (total 19 compounds) appeared to inhibit telomerase activity at 200µM concentration (figures 45 and 46).

4.3.2 Specificity of selected compounds for telomerase inhibition

Next the specificity of the compounds for inhibition of telomerase activity over other DNA polymerases (such as Taq polymerase) was assessed. It was necessary to determine if this inhibition of telomerase activity by the 19 novel compounds was a result of inhibition of the Taq polymerase within the TRAP assay. This was determined by examining the effect of the compounds on β actin PCR amplification. The results showed 15 of the 19 compounds which inhibited telomerase activity also appeared to inhibit the PCR reaction, but 4 compounds did not inhibit the PCR reaction (figures 47 and 48). This suggested that the 4 compounds (HE19, HE20, LSCMe and LSCEt) were able to inhibit telomerase specifically and these compounds were therefore selected for further analysis (figure 12).

Varying concentrations (200, 100, 50, and 25μM) of the 4 selected compounds (HE19, HE20, LSCMe and LSCEt) were incubated with the A549 cell lysate in order to determine the IC_{50} values. The mean IC_{50} values (the concentration at which 50% of the telomerase activity is lost) obtained for 4 selected compounds is shown in table 9. 3 of the compounds (HE19, HE20 and LSCMe) exhibited mean IC_{50} values above 100μM. LSCEt was found to be the most potent at 89μM.

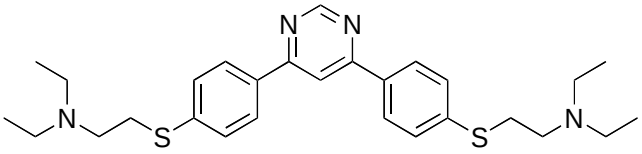
Structure activity relationships (SAR)

There are a limited number of structure activity relationships emerging from this study. Based upon the data provided in table X describing the ability of compounds to (i) inhibit telomerase specifically and (ii) inhibit polymerases in general (as indicated by inhibition of both telomerase and Taq) and (iii) no inhibition of polymerases, the following structure activity relationships are listed below.

SAR 1: Increasing the potency of selective telomerase inhibitors.

A comparison of the structures and activity of LSCet and LSCMe (figure 60) suggest that the addition of ethyl groups to the nitrogens on both 'arms' of the molecule (LSCet) may improve the potency of telomerase inhibition (figure 1). IC_{50} values for the inhibition of telomerase were 89 and 102 μM for LSCet and LSCMe respectively. The differences in potency are small but it suggests that modifications to this site may be useful to direct future synthesis of new molecules.

LSCet (telomerase specific inhibitor)



LSCMe (telomerase and taq inhibitor)

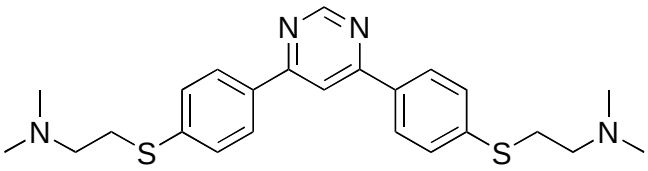
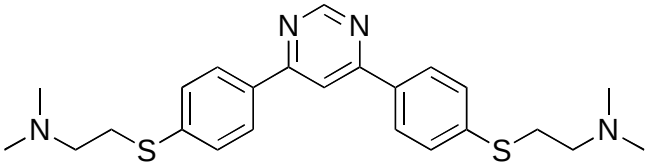


Figure 60 LSCMe and LSCet are both Telomerase specific but with varying potencies

SAR2: Selectivity for telomerase over Taq polymerase.

A comparison between the structures of LSCme (telomerase specific) and SJ45 (not specific for telomerase) demonstrates that the inclusion of an extra carbon into the side chain eliminates the selective inhibition of telomerase (figure 61). Modifications to the length of the side chain may therefore not be productive for future chemical synthesis.

LSCMe (telomerase specific inhibitor)



SJ45 (telomerase and taq inhibitor)

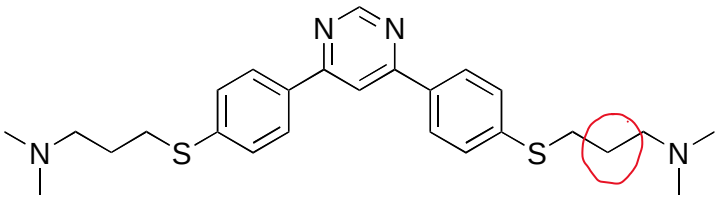
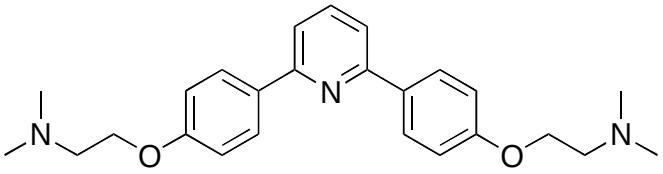


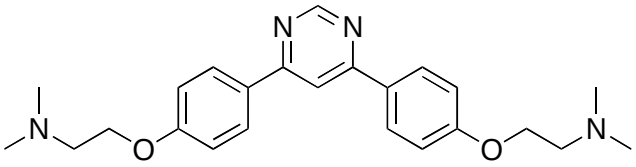
Figure 61 Extra CH_2 in the side arm of SJ45 destroys selectivity for telomerase

SAR3: Comparison between HE19 and HE 24 demonstrated that a pyridine ring but not a piperidine ring structure confers selectivity for telomerase (figure 62). This is also true for HE24 compared to HE25. The piperidine structure can nevertheless confer selectivity for telomerase (LSCMe above) but in this case the side arms contain an oxygen compared to a sulphur. The composition of the side arms together with the central ring structure are therefore important in terms of conferring selectivity for telomerase inhibition.

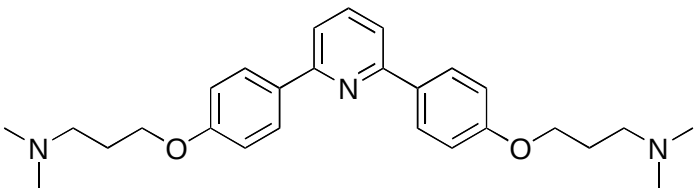
HE19 (telomerase specific inhibitor)



HE24 (inhibitor of both telomerase and taq polymerase)



HE20 (telomerase specific inhibitor)



HE25 (telomerase and taq inhibitor)

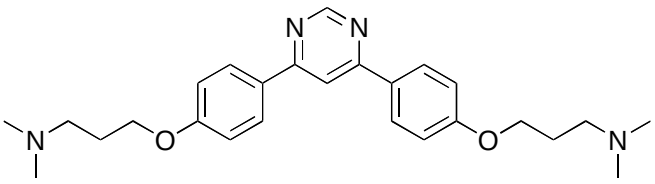
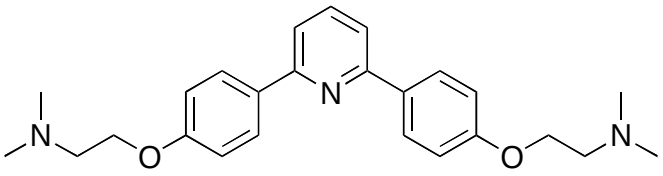


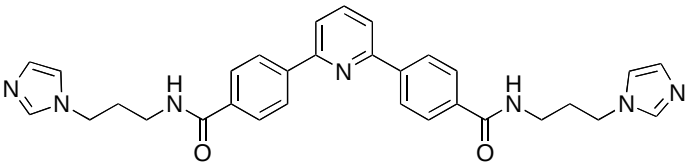
Figure 62 Piridine ring structure favours telomerase specific inhibition.

There are a number of additional structure activity relationships that confer lack of activity against both telomerase and taq polymerase. The majority of these are modifications to the side arms of the molecules and an example is shown below.

HE-19 (telomerase specific inhibitor)



AC-A (no inhibition of telomerase or taq)



4.3.3 Senecence assay

Senescence is an arrested state in which the cell remains viable but displays altered patterns of gene and protein expression. As described previously, the chromosomal ends are protected by the telomere which forms a cap at the end of the chromosomes. As well as the effect on the elongation of the telomere ends, telomerase also stabilizes the telomere through the additional stabilization of the telomere cap (Blasco 2002, Masutomi et al 2003, Sung et al 2005). It has been shown that interference with telomere architecture and maintenance can initiate senescence (Karlseder et al 2002, Leonetti et al 2004). Gowan et al (2001) showed that a pentacyclic acridine (RHPS4) was found to be a potent telomerase inhibitor in the TRAP assay and to cause irreversible cessation of growth after long term culture at noncytotoxic concentrations in cancer cells.

The ability of the selected compounds to induce cellular senescence was determined using the senescence assay. Cells lines selected were the PC3 and A549 cell lines. The methodology was developed by using arsenic which has been shown to induce DNA and chromosome damage. First of all, A549 cells and PC3 cells were incubated with arsenic and the selected compounds, and acute cytotoxic effects were assessed using the MTT assay as shown in figure 58. Subtoxic doses were then determined (tables 12 and 13). The PC3 cells and A549 cells were exposed to the sub toxic doses of arsenic and selected compounds for 5, 10 and 15 days, after which the plates were examined for the presence of senescent cells. There was absence of senescent cells after 5, 10 and 15 days in both the plates exposed to arsenic as well as to the selected compounds. This implied that the mechanism of cell toxicity of the compounds was not via the induction of cellular senescence.

4.3.4 Acute toxicity of selected compounds

A key goal in the development of telomerase inhibitors is the selection of compounds which have high telomerase activity inhibition at low concentrations, with relatively low concentrations for acute cytotoxicity. In order to further explore the effects of the 4 selected compounds as telomerase inhibitors, telomerase activity was first of all determined in a range of cell lines to determine telomerase positive and negative cell lines (figure 53). The acute toxicity of the selected compounds was assessed using the MTT assay in order to determine cell survival after drug exposure. As described previously, the A549 cell line was exposed to varying concentrations of the selected compounds. The response of the A549 cell line to the compounds after 2h and 96h exposures is shown in figure 56. The IC_{50} value for cell toxicity for each compound is shown in table 10. The IC_{50} values obtained for cell toxicity at 2h exposure in the A549 cell line are comparable to the values obtained in the cell free assay. Further experiments were performed to determine the effect of the compounds on telomerase negative cells (HUVEC) in order to determine if the acute toxicity effect of the compounds was influenced by the telomerase activity of the cell line. HUVEC cell line was exposed to varying concentrations of the selected compounds and the response to the compounds after 2h and 96h exposures is shown in figures 57. The IC_{50} values obtained at 2h exposure of the compounds to telomerase positive cells (A549) and telomerase negative cells (HUVEC) show that a higher concentration of HE19 and HE20 is required for acute cell toxicity in the telomerase positive cells. Similar concentrations of the compounds cause acute cell toxicity in both telomerase positive and negative cells lines at 96 hour exposure (table 11).

Overall, the 4 selected compounds appeared to inhibit telomerase in a cell free assay at similar concentrations required to cause acute cell toxicity at 2h exposure in

the A549 cells. This suggested that the selected compounds were telomerase inhibitors and caused acute cell toxicity. However, the mechanism of cell toxicity did not appear to be via the initiation of senescence.

.

Future work

The combination of telomerase inhibitors and traditional therapies may be the most effective way to target telomerase-positive tumors in the future (Ruden et al 2013). Currently, molecularly targeted therapies have become increasingly important in the treatment of advanced aggressive cancers (such as non small cell lung cancer and melanoma), as the traditional options of management have harmful side effects and are ineffective in the control of tumour progression (Rajanna et al 2015). Due to the role that telomeres play in the life cycle of cancers, telomerase and telomere-based therapeutics may be able to replace conventional treatments in the future (Ivancich et al, 2017). The undesirable side effects that conventional therapies elicit may be reduced or eliminated due to the specificity of these treatments. However, the non specificity of the compounds selected in this study, could be advantageous as multitargeting is a possible way tackling tumour heterogeneity.

Saliva testing offers advantages due to its non-invasive nature, the ease of collection, and storage of specimens, as well as the possibility of collecting multiple samples. However, saliva is a viscous fluid and difficulty in measuring precise volumes of viscous saliva samples can decrease the accuracy of analytical measurements. Most potential biomarkers are present in saliva at low concentrations such that any error in volume may give rise to large errors in the concentrations measured. Due to the low concentrations, assays with high sensitivity and low limit of detection are required, in addition to high selectivity and specificity (Ngamchuea et al. 2017). One of the challenges faced in research into salivary biomarkers is the lack of standardisation for saliva sample collection, processing and storage and effect of oral inflammatory conditions on samples collected (Rhadika et al. 2016). This had a possible effect in this study. In the future, a

reliable and standardised method of saliva collection would be the first step in validating saliva as a biomarker of disease.

5 REFERENCES

Allison, P., Franco, E., Black, M. and Feine, J. (1998a) The role of professional diagnostic delays in the prognosis of upper aerodigestive tract carcinoma. *Oral Oncol* 34 (2), 147-53.

Allison, P., Franco, E. and Feine, J. (1998b) Predictors of professional diagnostic delays for upper aerodigestive tract carcinoma. *Oral Oncol* 34 (2), 127-32.

Allison, P., Locker, D. and Feine, J. S. (1998c) The role of diagnostic delays in the prognosis of oral cancer: a review of the literature. *Oral Oncol* 34 (3), 161-70.

Anderson, A. R., Weaver, A. M., Cummings, P. T. and Quaranta, V. (2006) Tumor morphology and phenotypic evolution driven by selective pressure from the microenvironment. *Cell* 127 (5), 905-15.

Asai, A., Oshima, Y., Yamamoto, Y., Uochi, T. A., Kusaka, H., Akinaga, S., Yamashita, Y., Pongracz, K., Pruzan, R., Wunder, E., Piatyszek, M., Li, S., Chin, A. C., Harley, C. B. and Gryaznov, S. (2003) A novel telomerase template antagonist (GRN163) as a potential anticancer agent. *Cancer Res* 63 (14), 3931-9.

Azam, F., Mehta, S. and Harris, A. L. (2010) Mechanisms of resistance to antiangiogenesis therapy. *Eur J Cancer* 46 (8), 1323-32.

Bardoń, A. and Shugar, D. (1980) Properties of purified salivary ribonuclease, and salivary ribonuclease levels in children with cystic fibrosis and in heterozygous carriers. *Clin Chim Acta* 101 (1), 17-24.

Baudino, T. A. (2015) Targeted Cancer Therapy: The Next Generation of Cancer Treatment. *Curr Drug Discov Technol* 12 (1), 3-20.

Belcher, R., Hayes, K., Fedewa, S. and Chen, A. Y. (2014) Current treatment of head and neck squamous cell cancer. *J Surg Oncol* 110 (5), 551-74.

Blasco, M. A. (2002) Mouse models to study the role of telomeres in cancer, aging and DNA repair. *Eur J Cancer* 38 (17), 2222-8.

Blasco, M. A., Lee, H. W., Hande, M. P., Samper, E., Lansdorp, P. M., DePinho, R. A. and Greider, C. W. (1997) Telomere shortening and tumor formation by mouse cells lacking telomerase RNA. *Cell* 91 (1), 25-34.

Blumenschein, G. R., Mills, G. B. and Gonzalez-Angulo, A. M. (2012) Targeting the hepatocyte growth factor-cMET axis in cancer therapy. *J Clin Oncol* 30 (26), 3287-96.

Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S. and Wright, W. E. (1998) Extension of life-span by introduction of telomerase into normal human cells. *Science* 279 (5349), 349-52.

Bonner, J. A., Harari, P. M., Giralt, J., Azarnia, N., Shin, D. M., Cohen, R. B., Jones, C. U., Sur, R., Raben, D., Jassem, J., Ove, R., Kies, M. S., Baselga, J., Youssoufian, H., Amellal, N., Rowinsky, E. K. and Ang, K. K. (2006) Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med* 354 (6), 567-78.

Boyle, J. O., Mao, L., Brennan, J. A., Koch, W. M., Eisele, D. W., Saunders, J. R. and Sidransky, D. (1994) Gene mutations in saliva as molecular markers for head and neck squamous cell carcinomas. *Am J Surg* 168 (5), 429-32.

Brennan, J. A., Mao, L., Hruban, R. H., Boyle, J. O., Eby, Y. J., Koch, W. M., Goodman, S. N. and Sidransky, D. (1995) Molecular assessment of histopathological staging in squamous-cell carcinoma of the head and neck. *N Engl J Med* 332 (7), 429-35.

Broccoli, D., Smogorzewska, A., Chong, L. and de Lange, T. (1997) Human

telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. *Nat Genet* 17 (2), 231-5.

Broccoli, D., Young, J. W. and de Lange, T. (1995) Telomerase activity in normal and malignant hematopoietic cells. *Proc Natl Acad Sci U S A* 92 (20), 9082-6.

Brocklehurst, P., Kujan, O., Glenny, A. M., Oliver, R., Sloan, P., Ogden, G. and Shepherd, S. (2010) Screening programmes for the early detection and prevention of oral cancer. *Cochrane Database Syst Rev* (11), CD004150.

Brodeur, G. M. and Bagatell, R. (2014) Mechanisms of neuroblastoma regression. *Nat Rev Clin Oncol* 11 (12), 704-13.

Brown and Charles (2005) *Holland-Frei Manual of Cancer Medicine.* BC Decker.

Bruedigam, C. and Lane, S. W. (2016) Telomerase in hematologic malignancies. *Curr Opin Hematol* 23 (4), 346-53.

Burger, A. M. (2007) Highlights in experimental therapeutics. *Cancer Lett* 245 (1-2), 11-21.

Burger, A. M., Dai, F., Schultes, C. M., Reszka, A. P., Moore, M. J., Double, J. A. and Neidle, S. (2005) The G-quadruplex-interactive molecule BRACO-19 inhibits tumor growth, consistent with telomere targeting and interference with telomerase function. *Cancer Res* 65 (4), 1489-96.

Burger, A. M., Double, J. A. and Newell, D. R. (1997) Inhibition of telomerase activity by cisplatin in human testicular cancer cells. *Eur J Cancer* 33 (4), 638-44.

Buseman, C. M., Wright, W. E. and Shay, J. W. (2012) Is telomerase a viable target in cancer? *Mutat Res* 730 (1-2), 90-7.

Califano, J., Ahrendt, S. A., Meininger, G., Westra, W. H., Koch, W. M. and Sidransky, D. (1996a) Detection of telomerase activity in oral rinses from head and neck squamous cell carcinoma patients. *Cancer Res* 56 (24), 5720-2.

Califano, J., van der Riet, P., Westra, W., Nawroz, H., Clayman, G., Piantadosi, S., Corio, R., Lee, D., Greenberg, B., Koch, W. and Sidransky, D. (1996b) Genetic progression model for head and neck cancer: implications for field cancerization. *Cancer Res* 56 (11), 2488-92.

Cancer Research UK (2015) *Cancer incidence statistics.* Cancer Research UK. *http://www.cancerresearchuk.org/health-professional/cancer-statistics/incidence#heading-Zero* Accessed 23 November 2017.

Carney, D. N., Bunn, P. A., Gazdar, A. F., Pagan, J. A. and Minna, J. D. (1981) Selective growth in serum-free hormone-supplemented medium of tumor cells obtained by biopsy from patients with small cell carcinoma of the lung. *Proc Natl Acad Sci U S A* 78 (5), 3185-9.

Casadio, V., Bravaccini, S., Gunelli, R., Nanni, O., Zoli, W., Amadori, D., Calistri, D. and Silvestrini, R. (2009) Accuracy of urine telomerase activity to detect bladder cancer in symptomatic patients. *Int J Biol Markers* 24 (4), 253-7.

Cheng, Y. S., Rees, T. and Wright, J. (2014) A review of research on salivary biomarkers for oral cancer detection. *Clin Transl Med* 3 (1), 3.

Chiappori, A. A., Kolevska, T., Spigel, D. R., Hager, S., Rarick, M., Gadgeel, S., Blais, N., Von Pawel, J., Hart, L., Reck, M., Bassett, E., Burington, B. and Schiller, J. H. (2015) A randomized phase II study of the telomerase inhibitor imetelstat as maintenance therapy for advanced non-small-cell lung cancer. *Ann Oncol* 26 (2), 354-62.

Chou, W. C., Hawkins, A. L., Barrett, J. F., Griffin, C. A. and Dang, C. V. (2001) Arsenic inhibition of telomerase transcription leads to genetic instability. *J Clin Invest* 108 (10), 1541-7.

Cookson, J. C., Dai, F., Smith, V., Heald, R. A., Laughton, C. A., Stevens, M. F. and

Burger, A. M. (2005) Pharmacodynamics of the G-quadruplex-stabilizing telomerase inhibitor 3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl]acridinium methosulfate (RHPS4) in vitro: activity in human tumor cells correlates with telomere length and can be enhanced, or antagonized, with cytotoxic agents. *Mol Pharmacol* 68 (6), 1551-8.

Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B. and Bacchetti, S. (1992) Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J* 11 (5), 1921-9.

Cunningham, V. J., Markham, N., Shroyer, A. L. and Shroyer, K. R. (1998) Detection of telomerase expression in fine-needle aspirations and fluids. *Diagn Cytopathol* 18 (6), 431-6.

Curran, A. J., St Denis, K., Irish, J., Gullane, P. J., MacMillan, C. and Kamel-Reid, S. (1998) Telomerase activity in oral squamous cell carcinoma. *Arch Otolaryngol Head Neck Surg* 124 (7), 784-8.

Daley, T. and Darling, M. (2003) Nonsquamous cell malignant tumours of the oral cavity: an overview. *J Can Dent Assoc* 69 (9), 577-82.

Damm, K., Hemmann, U., Garin-Chesa, P., Hauel, N., Kauffmann, I., Priepke, H., Niestroj, C., Daiber, C., Enenkel, B., Guilliard, B., Lauritsch, I., Müller, E., Pascolo, E., Sauter, G., Pantic, M., Martens, U. M., Wenz, C., Lingner, J., Kraut, N., Rettig, W. J. and Schnapp, A. (2001) A highly selective telomerase inhibitor limiting human cancer cell proliferation. *EMBO J* 20 (24), 6958-68.

Dasari, S., Rajendra, W. and Valluru, L. (2014) Evaluation of soluble CD44 protein marker to distinguish the premalignant and malignant carcinoma cases in cervical cancer patients. *Med Oncol* 31 (9), 139.

de Lange, T. (2002) Protection of mammalian telomeres. *Oncogene* 21 (4), 532-40.

de Lange, T. (2005) Shelterin: the protein complex that shapes and safeguards human telomeres. *Genes Dev* 19 (18), 2100-10.

Decker, J. and Goldstein, J. C. (1982) Risk factors in head and neck cancer. *N Engl J Med* 306 (19), 1151-5.

Del Gaizo Moore, V., Schlis, K. D., Sallan, S. E., Armstrong, S. A. and Letai, A. (2008) BCL-2 dependence and ABT-737 sensitivity in acute lymphoblastic leukemia. *Blood* 111 (4), 2300-9.

Deng, J., Carlson, N., Takeyama, K., Dal Cin, P., Shipp, M. and Letai, A. (2007) BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. *Cancer Cell* 12 (2), 171-85.

Denis, F., Garaud, P., Bardet, E., Alfonsi, M., Sire, C., Germain, T., Bergerot, P., Rhein, B., Tortochaux, J. and Calais, G. (2004) Final results of the 94-01 French Head and Neck Oncology and Radiotherapy Group randomized trial comparing radiotherapy alone with concomitant radiochemotherapy in advanced-stage oropharynx carcinoma. *J Clin Oncol* 22 (1), 69-76.

Dikmen, Z. G., Wright, W. E., Shay, J. W. and Gryaznov, S. M. (2008) Telomerase targeted oligonucleotide thio-phosphoramidates in T24-luc bladder cancer cells. *J Cell Biochem* 104 (2), 444-52.

Dilley, R. L. and Greenberg, R. A. (2015) ALTernative Telomere Maintenance and Cancer. *Trends Cancer* 1 (2), 145-156.

Djojosubroto, M. W., Chin, A. C., Go, N., Schaetzlein, S., Manns, M. P., Gryaznov, S., Harley, C. B. and Rudolph, K. L. (2005) Telomerase antagonists GRN163 and GRN163L inhibit tumor growth and increase chemosensitivity of human

hepatoma. *Hepatology* 42 (5), 1127-36.

Doksani, Y., Wu, J. Y., de Lange, T. and Zhuang, X. (2013) Super-resolution fluorescence imaging of telomeres reveals TRF2-dependent T-loop formation. *Cell* 155 (2), 345-356.

Dominick, P. K., Keppler, B. R., Legassie, J. D., Moon, I. K. and Jarstfer, M. B. (2004) Nucleic acid-binding ligands identify new mechanisms to inhibit telomerase. *Bioorg Med Chem Lett* 14 (13), 3467-71.

Dragovich, T., Laheru, D., Dayyani, F., Bolejack, V., Smith, L., Seng, J., Burris, H., Rosen, P., Hidalgo, M., Ritch, P., Baker, A. F., Raghunand, N., Crowley, J. and Von Hoff, D. D. (2014) Phase II trial of vatalanib in patients with advanced or metastatic pancreatic adenocarcinoma after first-line gemcitabine therapy (PCRT O4-001). *Cancer Chemother Pharmacol* 74 (2), 379-87.

Drygin, D., Siddiqui-Jain, A., O'Brien, S., Schwaebe, M., Lin, A., Bliesath, J., Ho, C. B., Proffitt, C., Trent, K., Whitten, J. P., Lim, J. K., Von Hoff, D., Anderes, K. and Rice, W. G. (2009) Anticancer activity of CX-3543: a direct inhibitor of rRNA biogenesis. *Cancer Res* 69 (19), 7653-61.

Dziadziuszko, R. and Jassem, J. (2012) Epidermal growth factor receptor (EGFR) inhibitors and derived treatments. *Ann Oncol* 23 Suppl 10, x193-6.

Eichel, H. J., Conger, N. and Chernick, W. S. (1964) Acid and alkaline ribonucleases of human parotid, submaxillaryn and whole saliva. *Arch Biochem Biophys* 107, 197-208.

El-Kenawi, A. E. and El-Remessy, A. B. (2013) Angiogenesis inhibitors in cancer therapy: mechanistic perspective on classification and treatment rationales. *Br J Pharmacol* 170 (4), 712-29.

Epstein, J. B., Zhang, L. and Rosin, M. (2002) Advances in the diagnosis of oral

premalignant and malignant lesions. *J Can Dent Assoc* 68 (10), 617-21.

Fearon, E. R. and Vogelstein, B. (1990) A genetic model for colorectal tumorigenesis. *Cell* 61 (5), 759-67.

Feng, J., Funk, W. D., Wang, S. S., Weinrich, S. L., Avilion, A. A., Chiu, C. P., Adams, R. R., Chang, E., Allsopp, R. C. and Yu, J. (1995) The RNA component of human telomerase. *Science* 269 (5228), 1236-41.

Finn, R. S., Crown, J. P., Lang, I., Boer, K., Bondarenko, I. M., Kulyk, S. O., Ettl, J., Patel, R., Pinter, T., Schmidt, M., Shparyk, Y., Thummala, A. R., Voytko, N. L., Fowst, C., Huang, X., Kim, S. T., Randolph, S. and Slamon, D. J. (2015) The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study. *Lancet Oncol* 16 (1), 25-35.

Fleming, I. D., Phillips, J. L., Menck, H. R., Murphy, G. P. and Winchester, D. P. (1997) The National Cancer Data Base report on recent hospital cancer program progress toward complete American Joint Committee on Cancer/TNM staging. *Cancer* 80 (12), 2305-10.

Fogh, J., Fogh, J. M. and Orfeo, T. (1977) One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst* 59 (1), 221-6.

Forastiere, A. A. and Trotti, A. (1999) Radiotherapy and concurrent chemotherapy: a strategy that improves locoregional control and survival in oropharyngeal cancer. *J Natl Cancer Inst* 91 (24), 2065-6.

Forastiere, A. A., Trotti, A., Pfister, D. G. and Grandis, J. R. (2006) Head and neck cancer: recent advances and new standards of care. *J Clin Oncol* 24 (17),

2603-5.

Franzmann, E. J., Reategui, E. P., Pereira, L. H., Pedroso, F., Joseph, D., Allen, G. O., Hamilton, K., Reis, I., Duncan, R., Goodwin, W. J., Hu, J. J. and Lokeshwar, V. B. (2012) Salivary protein and solCD44 levels as a potential screening tool for early detection of head and neck squamous cell carcinoma. *Head Neck* 34 (5), 687-95.

Fujimori, J., Matsuo, T., Shimose, S., Kubo, T., Ishikawa, M., Yasunaga, Y. and Ochi, M. (2011) Antitumor effects of telomerase inhibitor TMPyP4 in osteosarcoma cell lines. *J Orthop Res* 29 (11), 1707-11.

Garden, A. S. (2001) Altered fractionation for head and neck cancer. *Oncology (Williston Park)* 15 (10), 1326-32, 1334; discussion 1334, 1339-41.

Gellert, G. C., Dikmen, Z. G., Wright, W. E., Gryaznov, S. and Shay, J. W. (2006) Effects of a novel telomerase inhibitor, GRN163L, in human breast cancer. *Breast Cancer Res Treat* 96 (1), 73-81.

Giard, D. J., Aaronson, S. A., Todaro, G. J., Arnstein, P., Kersey, J. H., Dosik, H. and Parks, W. P. (1973) In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J Natl Cancer Inst* 51 (5), 1417-23.

Gillison, M. L., Koch, W. M., Capone, R. B., Spafford, M., Westra, W. H., Wu, L., Zahurak, M. L., Daniel, R. W., Viglione, M., Symer, D. E., Shah, K. V. and Sidransky, D. (2000) Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst* 92 (9), 709-20.

Giri, S., Nieber, K., Acikgöz, A., Pavlica, S., Keller, M. and Bader, A. (2010) Telomerase activity and hepatic functions of rat embryonic liver progenitor cell in nanoscaffold-coated model bioreactor. *Mol Cell Biochem* 336 (1-2), 137-49.

Goldblatt, E. M., Erickson, P. A., Gentry, E. R., Gryaznov, S. M. and Herbert, B. S. (2009) Lipid-conjugated telomerase template antagonists sensitize resistant HER2-positive breast cancer cells to trastuzumab. *Breast Cancer Res Treat* 118 (1), 21-32.

Goodman, L. S. and Wintrobe, M. M. (1946) Nitrogen mustard therapy; use of methyl-bis (beta-chloroethyl) amine hydrochloride and tris (beta-chloroethyl) amine hydrochloride for Hodgkin's disease, lymphosarcoma, leukemia and certain allied and miscellaneous disorders. *J Am Med Assoc* 132, 126-32.

Gowan, S. M., Harrison, J. R., Patterson, L., Valenti, M., Read, M. A., Neidle, S. and Kelland, L. R. (2002) A G-quadruplex-interactive potent small-molecule inhibitor of telomerase exhibiting in vitro and in vivo antitumor activity. *Mol Pharmacol* 61 (5), 1154-62.

Gowan, S. M., Heald, R., Stevens, M. F. and Kelland, L. R. (2001) Potent inhibition of telomerase by small-molecule pentacyclic acridines capable of interacting with G-quadruplexes. *Mol Pharmacol* 60 (5), 981-8.

Grand, C. L., Han, H., Muñoz, R. M., Weitman, S., Von Hoff, D. D., Hurley, L. H. and Bearss, D. J. (2002) The cationic porphyrin TMPyP4 down-regulates c-MYC and human telomerase reverse transcriptase expression and inhibits tumor growth in vivo. *Mol Cancer Ther* 1 (8), 565-73.

Graveland, A. P., Bremmer, J. F., de Maaker, M., Brink, A., Cobussen, P., Zwart, M., Braakhuis, B. J., Bloemena, E., van der Waal, I., Leemans, C. R. and Brakenhoff, R. H. (2013) Molecular screening of oral precancer. *Oral Oncol* 49 (12), 1129-35.

Greider, C. W. and Blackburn, E. H. (1985) Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell* 43 (2 Pt 1), 405-13.

Griffith, J. D., Comeau, L., Rosenfield, S., Stansel, R. M., Bianchi, A., Moss, H. and de Lange, T. (1999) Mammalian telomeres end in a large duplex loop. *Cell* 97 (4), 503-14.

Group., B. D. W. (2001) Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* 69 (3), 89-95.

Gryaznov, S. M., Jackson, S., Dikmen, G., Harley, C., Herbert, B. S., Wright, W. E. and Shay, J. W. (2007) Oligonucleotide conjugate GRN163L targeting human telomerase as potential anticancer and antimetastatic agent. *Nucleosides Nucleotides Nucleic Acids* 26 (10-12), 1577-9.

Gunaratnam, M., Greciano, O., Martins, C., Reszka, A. P., Schultes, C. M., Morjani, H., Riou, J. F. and Neidle, S. (2007) Mechanism of acridine-based telomerase inhibition and telomere shortening. *Biochem Pharmacol* 74 (5), 679-89.

Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W. and Weinberg, R. A. (1999) Creation of human tumour cells with defined genetic elements. *Nature* 400 (6743), 464-8.

Haider, S. M., Parkinson, G. N. and Neidle, S. (2003) Structure of a G-quadruplex-ligand complex. *J Mol Biol* 326 (1), 117-25.

Hamilton, T. C., Young, R. C. and Ozols, R. F. (1984) Experimental model systems of ovarian cancer: applications to the design and evaluation of new treatment approaches. *Semin Oncol* 11 (3), 285-98.

Hanahan, D. and Weinberg, R. (2000) Hallmarks of cancer. *Cell* 100, 57-70.

Hanahan, D. and Weinberg, R. A. (2011) Hallmarks of cancer: the next generation. *Cell* 144 (5), 646-74.

Harley, C. B., Futcher, A. B. and Greider, C. W. (1990) Telomeres shorten during ageing of human fibroblasts. *Nature* 345 (6274), 458-60.

Harrington, L., McPhail, T., Mar, V., Zhou, W., Oulton, R., Bass, M. B., Arruda, I. and Robinson, M. O. (1997) A mammalian telomerase-associated protein. *Science* 275 (5302), 973-7.

Hastie, N. D., Dempster, M., Dunlop, M. G., Thompson, A. M., Green, D. K. and Allshire, R. C. (1990) Telomere reduction in human colorectal carcinoma and with ageing. *Nature* 346 (6287), 866-8.

Hayflick, L. (1997) Mortality and immortality at the cellular level. A review. *Biochemistry (Mosc)* 62 (11), 1180-90.

Hayflick, L. and Moorhead, P. S. (1961) The serial cultivation of human diploid cell strains. *Exp Cell Res* 25, 585-621.

Henson, J. D., Neumann, A. A., Yeager, T. R. and Reddel, R. R. (2002) Alternative lengthening of telomeres in mammalian cells. *Oncogene* 21 (4), 598-610.

Herbert, B., Pitts, A. E., Baker, S. I., Hamilton, S. E., Wright, W. E., Shay, J. W. and Corey, D. R. (1999) Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. *Proc Natl Acad Sci U S A* 96 (25), 14276-81.

Herbert, B. S., Gellert, G. C., Hochreiter, A., Pongracz, K., Wright, W. E., Zielinska, D., Chin, A. C., Harley, C. B., Shay, J. W. and Gryaznov, S. M. (2005) Lipid modification of GRN163, an N3'-->P5' thio-phosphoramidate oligonucleotide, enhances the potency of telomerase inhibition. *Oncogene* 24 (33), 5262-8.

Hermida, M., Ferreiro, M. C., Barral, S., Laredo, R., Castro, A. and Diz Dios, P. (2002) Detection of HCV RNA in saliva of patients with hepatitis C virus infection by using a highly sensitive test. *J Virol Methods* 101 (1-2), 29-35.

Hess, J. L. and Highsmith, W. E. (2002) Telomerase detection in body fluids. *Clin Chem* 48 (1), 18-24.

Hirvikoski, P., Tammi, R., Kumpulainen, E., Virtaniemi, J., Parkkinen, J. J., Tammi, M., Johansson, R., Agren, U., Karhunen, J. and Kosma, V. M. (1999) Irregular expression of hyaluronan and its CD44 receptor is associated with metastatic phenotype in laryngeal squamous cell carcinoma. *Virchows Arch* 434 (1), 37-44.

Hiyama, E., Hiyama, K., Yokoyama, T., Matsuura, Y., Piatyszek, M. A. and Shay, J. W. (1995) Correlating telomerase activity levels with human neuroblastoma outcomes. *Nat Med* 1 (3), 249-55.

Hiyama, E., Hiyama, K., Yokoyama, T. and Shay, J. W. (2001) Immunohistochemical detection of telomerase (hTERT) protein in human cancer tissues and a subset of cells in normal tissues. *Neoplasia* 3 (1), 17-26.

Hochhaus, A., Larson, R. A., Guilhot, F., Radich, J. P., Branford, S., Hughes, T. P., Baccarani, M., Deininger, M. W., Cervantes, F., Fujihara, S., Ortmann, C. E., Menssen, H. D., Kantarjian, H., O'Brien, S. G., Druker, B. J. and Investigators, I. (2017) Long-Term Outcomes of Imatinib Treatment for Chronic Myeloid Leukemia. *N Engl J Med* 376 (10), 917-927.

Holmstrup, P., Vedtofte, P., Reibel, J. and Stoltze, K. (2007) Oral premalignant lesions: is a biopsy reliable? *J Oral Pathol Med* 36 (5), 262-6.

Hunger, R. E., Kernland Lang, K., Markowski, C. J., Trachsel, S., Møller, M., Eriksen, J. A., Rasmussen, A. M., Braathen, L. R. and Gaudernack, G. (2011) Vaccination of patients with cutaneous melanoma with telomerase-specific peptides. *Cancer Immunol Immunother* 60 (11), 1553-64.

Hurwitz, H., Fehrenbacher, L., Novotny, W., Cartwright, T., Hainsworth, J., Heim, W., Berlin, J., Baron, A., Griffing, S., Holmgren, E., Ferrara, N., Fyfe, G., Rogers, B., Ross, R. and Kabbinavar, F. (2004) Bevacizumab plus irinotecan,

fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 350 (23), 2335-42.

Hwang, J. J., Kuruvilla, J., Mendelson, D., Pishvaian, M. J., Deeken, J. F., Siu, L. L., Berger, M. S., Viallet, J. and Marshall, J. L. (2010) Phase I dose finding studies of obatoclax (GX15-070), a small molecule pan-BCL-2 family antagonist, in patients with advanced solid tumors or lymphoma. *Clin Cancer Res* 16 (15), 4038-45.

Härle-Bachor, C. and Boukamp, P. (1996) Telomerase activity in the regenerative basal layer of the epidermis inhuman skin and in immortal and carcinoma-derived skin keratinocytes. *Proc Natl Acad Sci U S A* 93 (13), 6476-81.

Ivancich, M., Schrank, Z., Wojdyla, L., Leviskas, B., Kuckovic, A., Sanjali, A. and Puri, N. (2017) Treating Cancer by Targeting Telomeres and Telomerase. *Antioxidants (Basel)* 6 (1).

Izzo, J. G., Papadimitrakopoulou, V. A., Liu, D. D., den Hollander, P. L., Babenko, I. M., Keck, J., El-Naggar, A. K., Shin, D. M., Lee, J. J., Hong, W. K. and Hittelman, W. N. (2003) Cyclin D1 genotype, response to biochemoprevention, and progression rate to upper aerodigestive tract cancer. *J Natl Cancer Inst* 95 (3), 198-205.

Jafri, M. A., Ansari, S. A., Alqahtani, M. H. and Shay, J. W. (2016) Roles of telomeres and telomerase in cancer, and advances in telomerase-targeted therapies. *Genome Med* 8 (1), 69.

Jang, S. J., Chiba, I., Hirai, A., Hong, W. K. and Mao, L. (2001) Multiple oral squamous epithelial lesions: are they genetically related? *Oncogene* 20 (18), 2235-42.

Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F. and Jones, L. W. (1979)

Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol* 17 (1), 16-23.

Kaiser, M., De Cian, A., Sainlos, M., Renner, C., Mergny, J. L. and Teulade-Fichou, M. P. (2006) Neomycin-capped aromatic platforms: quadruplex DNA recognition and telomerase inhibition. *Org Biomol Chem* 4 (6), 1049-57.

Karlseder, J., Smogorzewska, A. and de Lange, T. (2002) Senescence induced by altered telomere state, not telomere loss. *Science* 295 (5564), 2446-9.

Keith, W. N., Bilsland, A., Evans, T. R. and Glasspool, R. M. (2002) Telomerase-directed molecular therapeutics. *Expert Rev Mol Med* 4 (10), 1-25.

Keith, W. N., Jeffry Evans, T. R. and Glasspool, R. M. (2001) Telomerase and cancer: time to move from a promising target to a clinical reality. *J Pathol* 195 (4), 404-14.

Kim, H. R., Christensen, R., Park, N. H., Sapp, P. and Kang, M. K. (2001) Elevated expression of hTERT is associated with dysplastic cell transformation during human oral carcinogenesis in situ. *Clin Cancer Res* 7 (10), 3079-86.

Kim, N. W. (1997) Clinical implications of telomerase in cancer. *Eur J Cancer* 33 (5), 781-6.

Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L. and Shay, J. W. (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science* 266 (5193), 2011-5.

Kim, S. H., Kaminker, P. and Campisi, J. (1999) TIN2, a new regulator of telomere length in human cells. *Nat Genet* 23 (4), 405-12.

Klewes, L., Vallente, R., Dupas, E., Brand, C., Grün, D., Guffei, A., Sathitruangsak, C., Awe, J. A., Kuzyk, A., Lichtensztejn, D., Tammur, P., Ilus, T., Tamm, A.,

Punab, M., Rubinger, M., Olujohungbe, A. and Mai, S. (2013) Three-dimensional Nuclear Telomere Organization in Multiple Myeloma. *Transl Oncol* 6 (6), 749-56.

Kowalski, L. P., Franco, E. L., Torloni, H., Fava, A. S., de Andrade Sobrinho, J., Ramos, G., Oliveira, B. V. and Curado, M. P. (1994) Lateness of diagnosis of oral and oropharyngeal carcinoma: factors related to the tumour, the patient and health professionals. *Eur J Cancer B Oral Oncol* 30B (3), 167-73.

Kurzrock, R., Sherman, S. I., Ball, D. W., Forastiere, A. A., Cohen, R. B., Mehra, R., Pfister, D. G., Cohen, E. E., Janisch, L., Nauling, F., Hong, D. S., Ng, C. S., Ye, L., Gagel, R. F., Frye, J., Müller, T., Ratain, M. J. and Salgia, R. (2011) Activity of XL184 (Cabozantinib), an oral tyrosine kinase inhibitor, in patients with medullary thyroid cancer. *J Clin Oncol* 29 (19), 2660-6.

Le, S., Moore, J. K., Haber, J. E. and Greider, C. W. (1999) RAD50 and RAD51 define two pathways that collaborate to maintain telomeres in the absence of telomerase. *Genetics* 152 (1), 143-52.

Leonetti, C., Amodei, S., D'Angelo, C., Rizzo, A., Benassi, B., Antonelli, A., Elli, R., Stevens, M. F., D'Incalci, M., Zupi, G. and Biroccio, A. (2004) Biological activity of the G-quadruplex ligand RHPS4 (3,11-difluoro-6,8,13-trimethyl-8H-quino[4,3,2-kl]acridinium methosulfate) is associated with telomere capping alteration. *Mol Pharmacol* 66 (5), 1138-46.

Li, J., Viallet, J. and Haura, E. B. (2008) A small molecule pan-Bcl-2 family inhibitor, GX15-070, induces apoptosis and enhances cisplatin-induced apoptosis in non-small cell lung cancer cells. *Cancer Chemother Pharmacol* 61 (3), 525-34.

Li, M. J., Kim, J., Li, S., Zaia, J., Yee, J. K., Anderson, J., Akkina, R. and Rossi, J. J.

(2005) Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. *Mol Ther* 12 (5), 900-9.

Li, Y., St John, M. A., Zhou, X., Kim, Y., Sinha, U., Jordan, R. C., Eisele, D., Abemayor, E., Elashoff, D., Park, N. H. and Wong, D. T. (2004) Salivary transcriptome diagnostics for oral cancer detection. *Clin Cancer Res* 10 (24), 8442-50.

Liao, P. H., Chang, Y. C., Huang, M. F., Tai, K. W. and Chou, M. Y. (2000) Mutation of p53 gene codon 63 in saliva as a molecular marker for oral squamous cell carcinomas. *Oral Oncol* 36 (3), 272-6.

Lipps, H. J. and Rhodes, D. (2009) G-quadruplex structures: in vivo evidence and function. *Trends Cell Biol* 19 (8), 414-22.

Liu, D., O'Connor, M. S., Qin, J. and Songyang, Z. (2004) Telosome, a mammalian telomere-associated complex formed by multiple telomeric proteins. *J Biol Chem* 279 (49), 51338-42.

Liu, J. P., Chen, W., Schwarer, A. P. and Li, H. (2010) Telomerase in cancer immunotherapy. *Biochim Biophys Acta* 1805 (1), 35-42.

Longchampt, E., Lebret, T., Molinie, V., Bieche, I., Botto, H. and Lidereau, R. (2003) [Detection of telomerase activity by semi-quantitative and in situ assays and quantification of hTERT expression in bladder carcinomas]. *Prog Urol* 13 (2), 238-45.

Lopes, V., Murray, P., Williams, H., Woodman, C., Watkinson, J. and Robinson, M. (2011) Squamous cell carcinoma of the oral cavity rarely harbours oncogenic human papillomavirus. *Oral Oncol* 47 (8), 698-701.

Lu, W., Zhang, Y., Liu, D., Songyang, Z. and Wan, M. (2013) Telomeres-structure, function, and regulation. *Exp Cell Res* 319 (2), 133-41.

Lu, Y., Gu, J., Jin, D., Gao, Y. and Yuan, M. (2011) Inhibition of telomerase activity by HDV ribozyme in cancers. *J Exp Clin Cancer Res* 30, 1.

Magnes, T., Egle, A., Greil, R. and Melchardt, T. (2017) Update on squamous cell carcinoma of the head and neck: ASCO annual meeting 2017. *Memo* 10 (4), 220-223.

Maier, H., Dietz, A., Gewelke, U., Heller, W. D. and Weidauer, H. (1992) Tobacco and alcohol and the risk of head and neck cancer. *Clin Investig* 70 (3-4), 320-7.

Mao, L., El-Naggar, A. K., Fan, Y. H., Lee, J. S., Lippman, S. M., Kayser, S., Lotan, R. and Hong, W. K. (1996) Telomerase activity in head and neck squamous cell carcinoma and adjacent tissues. *Cancer Res* 56 (24), 5600-4.

Mao, L., Hong, W. K. and Papadimitrakopoulou, V. A. (2004) Focus on head and neck cancer. *Cancer Cell* 5 (4), 311-6.

Markopoulos, A. K., Michailidou, E. Z. and Tzimagiorgis, G. (2010) Salivary markers for oral cancer detection. *Open Dent J* 4, 172-8.

Masutomi, K. and Hahn, W. C. (2003) Telomerase and tumorigenesis. *Cancer Lett* 194 (2), 163-72.

McClintock, B. (1941) The Stability of Broken Ends of Chromosomes in Zea Mays. *Genetics* 26 (2), 234-82.

McGurk, M., Chan, C., Jones, J., O'regan, E. and Sherriff, M. (2005) Delay in diagnosis and its effect on outcome in head and neck cancer. *Br J Oral Maxillofac Surg* 43 (4), 281-4.

Melana, S. M., Holland, J. F. and Pogo, B. G. (1998) Inhibition of cell growth and

telomerase activity of breast cancer cells in vitro by 3'-azido-3'-deoxythymidine. *Clin Cancer Res* 4 (3), 693-6.

Melissourgos, N., Kastrinakis, N. G., Davilas, I., Foukas, P., Farmakis, A. and Lykourinas, M. (2003) Detection of human telomerase reverse transcriptase mRNA in urine of patients with bladder cancer: evaluation of an emerging tumor marker. *Urology* 62 (2), 362-7.

Meyerson, M., Counter, C. M., Eaton, E. N., Ellisen, L. W., Steiner, P., Caddle, S. D., Ziaugra, L., Beijersbergen, R. L., Davidoff, M. J., Liu, Q., Bacchetti, S., Haber, D. A. and Weinberg, R. A. (1997) hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell* 90 (4), 785-95.

Mikami-Terao, Y., Akiyama, M., Yuza, Y., Yanagisawa, T., Yamada, O., Kawano, T., Agawa, M., Ida, H. and Yamada, H. (2009) Antitumor activity of TMPyP4 interacting G-quadruplex in retinoblastoma cell lines. *Exp Eye Res* 89 (2), 200-8.

Miller, K., Wang, M., Gralow, J., Dickler, M., Cobleigh, M., Perez, E. A., Shenkier, T., Cella, D. and Davidson, N. E. (2007) Paclitaxel plus bevacizumab versus paclitaxel alone for metastatic breast cancer. *N Engl J Med* 357 (26), 2666-76.

Mishra, A. and Verma, M. (2010) Cancer biomarkers: are we ready for the prime time? *Cancers (Basel)* 2 (1), 190-208.

Mitchell, J. R., Cheng, J. and Collins, K. (1999) A box H/ACA small nucleolar RNA-like domain at the human telomerase RNA 3' end. *Mol Cell Biol* 19 (1), 567-76.

Mitsudomi, T., Morita, S., Yatabe, Y., Negoro, S., Okamoto, I., Tsurutani, J., Seto, T., Satouchi, M., Tada, H., Hirashima, T., Asami, K., Katakami, N., Takada, M.,

Yoshioka, H., Shibata, K., Kudoh, S., Shimizu, E., Saito, H., Toyooka, S., Nakagawa, K., Fukuoka, M. and Group, W. J. O. (2010) Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol* 11 (2), 121-8.

Miyazaki, T., Pan, Y., Joshi, K., Purohit, D., Hu, B., Demir, H., Mazumder, S., Okabe, S., Yamori, T., Viapiano, M., Shin-ya, K., Seimiya, H. and Nakano, I. (2012) Telomestatin impairs glioma stem cell survival and growth through the disruption of telomeric G-quadruplex and inhibition of the proto-oncogene, c-Myb. *Clin Cancer Res* 18 (5), 1268-80.

Murukesh, N., Dive, C. and Jayson, G. C. (2010) Biomarkers of angiogenesis and their role in the development of VEGF inhibitors. *Br J Cancer* 102 (1), 8-18.

Mutirangura, A., Supiyaphun, P., Trirekapan, S., Sriuranpong, V., Sakuntabhai, A., Yenrudi, S. and Voravud, N. (1996) Telomerase activity in oral leukoplakia and head and neck squamous cell carcinoma. *Cancer Res* 56 (15), 3530-3.

Naasani, I., Seimiya, H., Yamori, T. and Tsuruo, T. (1999) FJ5002: a potent telomerase inhibitor identified by exploiting the disease-oriented screening program with COMPARE analysis. *Cancer Res* 59 (16), 4004-11.

Nakamura, T. M., Morin, G. B., Chapman, K. B., Weinrich, S. L., Andrews, W. H., Lingner, J., Harley, C. B. and Cech, T. R. (1997) Telomerase catalytic subunit homologs from fission yeast and human. *Science* 277 (5328), 955-9.

Nakayama, J., Tahara, H., Tahara, E., Saito, M., Ito, K., Nakamura, H., Nakanishi, T., Ide, T. and Ishikawa, F. (1998) Telomerase activation by hTRT in human normal fibroblasts and hepatocellular carcinomas. *Nat Genet* 18 (1), 65-8.

Neal, A. and Hoskin, P. (2009) *Clinical Oncology - basic principles and practice. 4th*

edition.

Neidle, S. (2010) Human telomeric G-quadruplex: the current status of telomeric G-quadruplexes as therapeutic targets in human cancer. *FEBS J* 277 (5), 1118-25.

Nemunaitis, J., Tong, A. W., Nemunaitis, M., Senzer, N., Phadke, A. P., Bedell, C., Adams, N., Zhang, Y. A., Maples, P. B., Chen, S., Pappen, B., Burke, J., Ichimaru, D., Urata, Y. and Fujiwara, T. (2010) A phase I study of telomerase-specific replication competent oncolytic adenovirus (telomelysin) for various solid tumors. *Mol Ther* 18 (2), 429-34.

Neville, B. W. and Day, T. A. (2002) Oral cancer and precancerous lesions. *CA Cancer J Clin* 52 (4), 195-215.

Ngamchuea, K., Chaisiwamongkhol, K., Batchelor-McAuley, C. and Compton, R. G. (2017) Chemical analysis in saliva and the search for salivary biomarkers - a tutorial review. *Analyst* 143 (1), 81-99.

Noreen, F., Heinrich, J. and Moelling, K. (2009) Antitumor activity of small double-stranded oligodeoxynucleotides targeting telomerase RNA in malignant melanoma cells. *Oligonucleotides* 19 (2), 169-78.

Novello, S., Scagliotti, G. V., Rosell, R., Socinski, M. A., Brahmer, J., Atkins, J., Pallares, C., Burgess, R., Tye, L., Selaru, P., Wang, E., Chao, R. and Govindan, R. (2009) Phase II study of continuous daily sunitinib dosing in patients with previously treated advanced non-small cell lung cancer. *Br J Cancer* 101 (9), 1543-8.

Nowell, P. C. (1976) The clonal evolution of tumor cell populations. *Science* 194 (4260), 23-8.

O'Toole, C. M., Povey, S., Hepburn, P. and Franks, L. M. (1983) Identity of some

human bladder cancer cell lines. *Nature* 301 (5899), 429-30.

Ohyashiki, K., Ohyashiki, J. H., Nishimaki, J., Toyama, K., Ebihara, Y., Kato, H., Wright, W. E. and Shay, J. W. (1997) Cytological detection of telomerase activity using an in situ telomeric repeat amplification protocol assay. *Cancer Res* 57 (11), 2100-3.

Olaussen, K. A., Dubrana, K., Domont, J., Spano, J. P., Sabatier, L. and Soria, J. C. (2006) Telomeres and telomerase as targets for anticancer drug development. *Crit Rev Oncol Hematol* 57 (3), 191-214.

Ouellette, M. M., Wright, W. E. and Shay, J. W. (2011) Targeting telomerase-expressing cancer cells. *J Cell Mol Med* 15 (7), 1433-42.

Palm, W. and de Lange, T. (2008) How shelterin protects mammalian telomeres. *Annu Rev Genet* 42, 301-34.

Park, N. J., Li, Y., Yu, T., Brinkman, B. M. and Wong, D. T. (2006) Characterization of RNA in saliva. *Clin Chem* 52 (6), 988-94.

Partridge, M. (2000a) Oral cancer: 1. The genetic basis of the disease. *Dent Update* 27 (5), 242-8.

Partridge, M. (2000b) Oral cancer: 1. The genetic basis of the disease. *Dent Update* 27 (5), 242-8.

Partridge, M. (2000c) Oral cancer: 2. Clinical presentation and use of new knowledge about the biology of cancer to establish why tumours may recur. *Dent Update* 27 (6), 288-94.

Partridge, M., Pateromichelakis, S., Phillips, E., Emilion, G. G., A'Hern, R. P. and Langdon, J. D. (2000) A case-control study confirms that microsatellite assay can identify patients at risk of developing oral squamous cell carcinoma within a field of cancerization. *Cancer Res* 60 (14), 3893-8.

Pascolo, E., Wenz, C., Lingner, J., Hauel, N., Priepke, H., Kauffmann, I., Garin-Chesa, P., Rettig, W. J., Damm, K. and Schnapp, A. (2002) Mechanism of human telomerase inhibition by BIBR1532, a synthetic, non-nucleosidic drug candidate. *J Biol Chem* 277 (18), 15566-72.

Patel, D. J., Phan, A. T. and Kuryavyi, V. (2007) Human telomere, oncogenic promoter and 5'-UTR G-quadruplexes: diverse higher order DNA and RNA targets for cancer therapeutics. *Nucleic Acids Res* 35 (22), 7429-55.

Peifer, M., Hertwig, F., Roels, F., Dreidax, D., Gartlgruber, M., Menon, R., Krämer, A., Roncaioli, J. L., Sand, F., Heuckmann, J. M., Ikram, F., Schmidt, R., Ackermann, S., Engesser, A., Kahlert, Y., Vogel, W., Altmüller, J., Nürnberg, P., Thierry-Mieg, J., Thierry-Mieg, D., Mariappan, A., Heynck, S., Mariotti, E., Henrich, K. O., Gloeckner, C., Bosco, G., Leuschner, I., Schweiger, M. R., Savelyeva, L., Watkins, S. C., Shao, C., Bell, E., Höfer, T., Achter, V., Lang, U., Theissen, J., Volland, R., Saadati, M., Eggert, A., de Wilde, B., Berthold, F., Peng, Z., Zhao, C., Shi, L., Ortmann, M., Büttner, R., Perner, S., Hero, B., Schramm, A., Schulte, J. H., Herrmann, C., O'Sullivan, R. J., Westermann, F., Thomas, R. K. and Fischer, M. (2015) Telomerase activation by genomic rearrangements in high-risk neuroblastoma. *Nature* 526 (7575), 700-4.

Pelengaris, S. and Khan, M. (2013) *The molecular biology of cancer: a bridge from bench to bedside.* Vol. 2nd. Chilchester: Wiley.

Perry, P. J., Read, M. A., Davies, R. T., Gowan, S. M., Reszka, A. P., Wood, A. A., Kelland, L. R. and Neidle, S. (1999) 2,7-Disubstituted amidofluorenone derivatives as inhibitors of human telomerase. *J Med Chem* 42 (14), 2679-84.

Petti, S. (2009) Lifestyle risk factors for oral cancer. *Oral Oncol* 45 (4-5), 340-50.

Phatak, P. and Burger, A. M. (2007) Telomerase and its potential for therapeutic

intervention. *Br J Pharmacol* 152 (7), 1003-11.

Phatak, P., Cookson, J. C., Dai, F., Smith, V., Gartenhaus, R. B., Stevens, M. F. and Burger, A. M. (2007) Telomere uncapping by the G-quadruplex ligand RHPS4 inhibits clonogenic tumour cell growth in vitro and in vivo consistent with a cancer stem cell targeting mechanism. *Br J Cancer* 96 (8), 1223-33.

Pomerantz, R. G. and Grandis, J. R. (2003) The role of epidermal growth factor receptor in head and neck squamous cell carcinoma. *Curr Oncol Rep* 5 (2), 140-6.

Prime, S. S., Nixon, S. V., Crane, I. J., Stone, A., Matthews, J. B., Maitland, N. J., Remnant, L., Powell, S. K., Game, S. M. and Scully, C. (1990) The behaviour of human oral squamous cell carcinoma in cell culture. *J Pathol* 160 (3), 259-69.

Pérez-Galán, P., Roué, G., Villamor, N., Campo, E. and Colomer, D. (2007) The BH3-mimetic GX15-070 synergizes with bortezomib in mantle cell lymphoma by enhancing Noxa-mediated activation of Bak. *Blood* 109 (10), 4441-9.

Radhika, T., Jeddy, N., Nithya, S. and Muthumeenakshi, R. M. (2016) Salivary biomarkers in oral squamous cell carcinoma - An insight. *J Oral Biol Craniofac Res* 6 (Suppl 1), S51-S54.

Rajanna, S., Rastogi, I., Wojdyla, L., Furo, H., Kulesza, A., Lin, L., Sheu, B., Frakes, M., Ivanovich, M. and Puri, N. (2015) Current Molecularly Targeting Therapies in NSCLC and Melanoma. *Anticancer Agents Med Chem* 15 (7), 856-68.

Rasheed, S., Nelson-Rees, W. A., Toth, E. M., Arnstein, P. and Gardner, M. B. (1974) Characterization of a newly derived human sarcoma cell line (HT-1080). *Cancer* 33 (4), 1027-33.

Reck, M. and Crinò, L. (2009) Advances in anti-VEGF and anti-EGFR therapy for

advanced non-small cell lung cancer. *Lung Cancer* 63 (1), 1-9.

Reck, M., von Pawel, J., Zatloukal, P., Ramlau, R., Gorbounova, V., Hirsh, V., Leighl, N., Mezger, J., Archer, V., Moore, N. and Manegold, C. (2009) Phase III trial of cisplatin plus gemcitabine with either placebo or bevacizumab as first-line therapy for nonsquamous non-small-cell lung cancer: AVAil. *J Clin Oncol* 27 (8), 1227-34.

Reed, J., Gunaratnam, M., Beltran, M., Reszka, A. P., Vilar, R. and Neidle, S. (2008) TRAP-LIG, a modified telomere repeat amplification protocol assay to quantitate telomerase inhibition by small molecules. *Anal Biochem* 380 (1), 99-105.

Ritz, J. M., Kühle, O., Riethdorf, S., Sipos, B., Deppert, W., Englert, C. and Günes, C. (2005) A novel transgenic mouse model reveals humanlike regulation of an 8-kbp human TERT gene promoter fragment in normal and tumor tissues. *Cancer Res* 65 (4), 1187-96.

Rosell, R., Carcereny, E., Gervais, R., Vergnenegre, A., Massuti, B., Felip, E., Palmero, R., Garcia-Gomez, R., Pallares, C., Sanchez, J. M., Porta, R., Cobo, M., Garrido, P., Longo, F., Moran, T., Insa, A., De Marinis, F., Corre, R., Bover, I., Illiano, A., Dansin, E., de Castro, J., Milella, M., Reguart, N., Altavilla, G., Jimenez, U., Provencio, M., Moreno, M. A., Terrasa, J., Muñoz-Langa, J., Valdivia, J., Isla, D., Domine, M., Molinier, O., Mazieres, J., Baize, N., Garcia-Campelo, R., Robinet, G., Rodriguez-Abreu, D., Lopez-Vivanco, G., Gebbia, V., Ferrera-Delgado, L., Bombaron, P., Bernabe, R., Bearz, A., Artal, A., Cortesi, E., Rolfo, C., Sanchez-Ronco, M., Drozdowskyj, A., Queralt, C., de Aguirre, I., Ramirez, J. L., Sanchez, J. J., Molina, M. A., Taron, M., Paz-Ares, L. and Toracica, S. L. C. G. i. c. w. G. F. d. P.-C. a. A. I. O. (2012)

Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. *Lancet Oncol* 13 (3), 239-46.

Rosin, M. P., Cheng, X., Poh, C., Lam, W. L., Huang, Y., Lovas, J., Berean, K., Epstein, J. B., Priddy, R., Le, N. D. and Zhang, L. (2000) Use of allelic loss to predict malignant risk for low-grade oral epithelial dysplasia. *Clin Cancer Res* 6 (2), 357-62.

Rossi, M. J., Sundararaj, K., Koybasi, S., Phillips, M. S., Szulc, Z. M., Bielawska, A., Day, T. A., Obeid, L. M., Hannun, Y. A. and Ogretmen, B. (2005) Inhibition of growth and telomerase activity by novel cationic ceramide analogs with high solubility in human head and neck squamous cell carcinoma cells. *Otolaryngol Head Neck Surg* 132 (1), 55-62.

Ruden, M. and Puri, N. (2013) Novel anticancer therapeutics targeting telomerase. *Cancer Treat Rev* 39 (5), 444-56.

Röth, A., Harley, C. B. and Baerlocher, G. M. (2010a) Imetelstat (GRN163L)--telomerase-based cancer therapy. *Recent Results Cancer Res* 184, 221-34.

Röth, A., Harley, C. B. and Baerlocher, G. M. (2010b) Imetelstat (GRN163L)--telomerase-based cancer therapy. *Recent Results Cancer Res* 184, 221-34.

Sager, R. (1991) Senescence as a mode of tumor suppression. *Environ Health Perspect* 93, 59-62.

Salvati, E., Leonetti, C., Rizzo, A., Scarsella, M., Mottolese, M., Galati, R., Sperduti, I., Stevens, M. F., D'Incalci, M., Blasco, M., Chiorino, G., Bauwens, S., Horard, B., Gilson, E., Stoppacciaro, A., Zupi, G. and Biroccio, A. (2007a) Telomere damage induced by the G-quadruplex ligand RHPS4 has an

antitumor effect. *J Clin Invest* 117 (11), 3236-47.

Salvati, E., Leonetti, C., Rizzo, A., Scarsella, M., Mottolese, M., Galati, R., Sperduti, I., Stevens, M. F., D'Incalci, M., Blasco, M., Chiorino, G., Bauwens, S., Horard, B., Gilson, E., Stoppacciaro, A., Zupi, G. and Biroccio, A. (2007b) Telomere damage induced by the G-quadruplex ligand RHPS4 has an antitumor effect. *J Clin Invest* 117 (11), 3236-47.

Sankaranarayanan, R., Ramadas, K., Thara, S., Muwonge, R., Thomas, G., Anju, G. and Mathew, B. (2013) Long term effect of visual screening on oral cancer incidence and mortality in a randomized trial in Kerala, India. *Oral Oncol* 49 (4), 314-21.

Santoro, A., Rimassa, L., Borbath, I., Daniele, B., Salvagni, S., Van Laethem, J. L., Van Vlierberghe, H., Trojan, J., Kolligs, F. T., Weiss, A., Miles, S., Gasbarrini, A., Lencioni, M., Cicalese, L., Sherman, M., Gridelli, C., Buggisch, P., Gerken, G., Schmid, R. M., Boni, C., Personeni, N., Hassoun, Z., Abbadessa, G., Schwartz, B., Von Roemeling, R., Lamar, M. E., Chen, Y. and Porta, C. (2013) Tivantinib for second-line treatment of advanced hepatocellular carcinoma: a randomised, placebo-controlled phase 2 study. *Lancet Oncol* 14 (1), 55-63.

Sausville, E. A. and Feigal, E. (1999) Evolving approaches to cancer drug discovery and development at the National Cancer Institute, USA. *Ann Oncol* 10 (11), 1287-91.

Schöffski, P., Garcia, J. A., Stadler, W. M., Gil, T., Jonasch, E., Tagawa, S. T., Smitt, M., Yang, X., Oliner, K. S., Anderson, A., Zhu, M. and Kabbinavar, F. (2011) A phase II study of the efficacy and safety of AMG 102 in patients with metastatic renal cell carcinoma. *BJU Int* 108 (5), 679-86.

Seiwert, T. Y., Jagadeeswaran, R., Faoro, L., Janamanchi, V., Nallasura, V., El Dinali, M., Yala, S., Kanteti, R., Cohen, E. E., Lingen, M. W., Martin, L., Krishnaswamy, S., Klein-Szanto, A., Christensen, J. G., Vokes, E. E. and Salgia, R. (2009) The MET receptor tyrosine kinase is a potential novel therapeutic target for head and neck squamous cell carcinoma. *Cancer Res* 69 (7), 3021-31.

Sekaran, V., Soares, J. and Jarstfer, M. B. (2014) Telomere maintenance as a target for drug discovery. *J Med Chem* 57 (3), 521-38.

Shalaby, T., Fiaschetti, G., Nagasawa, K., Shin-ya, K., Baumgartner, M. and Grotzer, M. (2013) G-quadruplexes as potential therapeutic targets for embryonal tumors. *Molecules* 18 (10), 12500-37.

Shay, J. W. and Bacchetti, S. (1997) A survey of telomerase activity in human cancer. *Eur J Cancer* 33 (5), 787-91.

Shay, J. W. and Wright, W. E. (1996) The reactivation of telomerase activity in cancer progression. *Trends Genet* 12 (4), 129-31.

Shay, J. W. and Wright, W. E. (2006) Telomerase therapeutics for cancer: challenges and new directions. *Nat Rev Drug Discov* 5 (7), 577-84.

Shi, D. F., Wheelhouse, R. T., Sun, D. and Hurley, L. H. (2001) Quadruplex-interactive agents as telomerase inhibitors: synthesis of porphyrins and structure-activity relationship for the inhibition of telomerase. *J Med Chem* 44 (26), 4509-23.

Shi, S., Gao, S., Cao, T., Liu, J., Gao, X., Hao, J., Lv, C., Huang, H., Xu, J. and Yao, T. (2013) Targeting human telomeric G-quadruplex DNA and inhibition of telomerase activity with [(dmb)2Ru(obip)Ru(dmb)2](4+). *PLoS One* 8 (12), e84419.

Shin-ya, K., Wierzba, K., Matsuo, K., Ohtani, T., Yamada, Y., Furihata, K., Hayakawa, Y. and Seto, H. (2001) Telomestatin, a novel telomerase inhibitor from Streptomyces anulatus. *J Am Chem Soc* 123 (6), 1262-3.

Silverman, S. (1988) Early diagnosis of oral cancer. *Cancer* 62 (8 Suppl), 1796-9.

SLAUGHTER, D. P., SOUTHWICK, H. W. and SMEJKAL, W. (1953) Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin. *Cancer* 6 (5), 963-8.

Smith, S., Giriat, I., Schmitt, A. and de Lange, T. (1998) Tankyrase, a poly (ADP-ribose) polymerase at human telomeres. *Science* 282 (5393), 1484-7.

Sobin, L., Gospodarowicz, M. and Wittekind, C. (2009) *TNM Classification of malignant tumours.* 7th ed. 2009 edition. Chichester, West Sussex, UK; Hoboken, NJ : Wiley-Blackwell, 2010.

Socinski, M. A. (2008) The current status and evolving role of sunitinib in non-small cell lung cancer. *J Thorac Oncol* 3 (6 Suppl 2), S119-23.

Sommerfeld, H. J., Meeker, A. K., Piatyszek, M. A., Bova, G. S., Shay, J. W. and Coffey, D. S. (1996) Telomerase activity: a prevalent marker of malignant human prostate tissue. *Cancer Res* 56 (1), 218-22.

Soyer, H. P., Massone, C., Ferrara, G. and Argenziano, G. (2005) Limitations of histopathologic analysis in the recognition of melanoma: a plea for a combined diagnostic approach of histopathologic and dermoscopic evaluation. *Arch Dermatol* 141 (2), 209-11.

Spafford, M. F., Koch, W. M., Reed, A. L., Califano, J. A., Xu, L. H., Eisenberger, C. F., Yip, L., Leong, P. L., Wu, L., Liu, S. X., Jerónimo, C., Westra, W. H. and Sidransky, D. (2001) Detection of head and neck squamous cell carcinoma among exfoliated oral mucosal cells by microsatellite analysis. *Clin Cancer*

Res 7 (3), 607-12.

Stegmeier, F., Warmuth, M., Sellers, W. R. and Dorsch, M. (2010) Targeted cancer therapies in the twenty-first century: lessons from imatinib. *Clin Pharmacol Ther* 87 (5), 543-52.

Stewart, B. and Wild, C. (2014) *World Cancer Report 2014.* International Agency for Research on Cancer, World Health Organisation. *http://publications.iarc.fr/Non-Series-Publications/World-Cancer-Reports/World-Cancer-Report-2014*

Strahl, C. and Blackburn, E. H. (1996) Effects of reverse transcriptase inhibitors on telomere length and telomerase activity in two immortalized human cell lines. *Mol Cell Biol* 16 (1), 53-65.

Strickler, J. H. and Hurwitz, H. I. (2012) Bevacizumab-based therapies in the first-line treatment of metastatic colorectal cancer. *Oncologist* 17 (4), 513-24.

Sumida, T., Sogawa, K., Hamakawa, H., Sugita, A., Tanioka, H. and Ueda, N. (1998) Detection of telomerase activity in oral lesions. *J Oral Pathol Med* 27 (3), 111-5.

Sun, D., Thompson, B., Cathers, B. E., Salazar, M., Kerwin, S. M., Trent, J. O., Jenkins, T. C., Neidle, S. and Hurley, L. H. (1997) Inhibition of human telomerase by a G-quadruplex-interactive compound. *J Med Chem* 40 (14), 2113-6.

Sung, Y. H., Choi, Y. S., Cheong, C. and Lee, H. W. (2005) The pleiotropy of telomerase against cell death. *Mol Cells* 19 (3), 303-9.

Sutlu, T. and Alici, E. (2009) Natural killer cell-based immunotherapy in cancer: current insights and future prospects. *J Intern Med* 266 (2), 154-81.

Sánchez-Martínez, C., Gelbert, L. M., Lallena, M. J. and de Dios, A. (2015) Cyclin

dependent kinase (CDK) inhibitors as anticancer drugs. *Bioorg Med Chem Lett* 25 (17), 3420-35.

Tabor, M. P., Brakenhoff, R. H., van Houten, V. M., Kummer, J. A., Snel, M. H., Snijders, P. J., Snow, G. B., Leemans, C. R. and Braakhuis, B. J. (2001) Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications. *Clin Cancer Res* 7 (6), 1523-32.

Tauchi, T., Shin-Ya, K., Sashida, G., Sumi, M., Nakajima, A., Shimamoto, T., Ohyashiki, J. H. and Ohyashiki, K. (2003) Activity of a novel G-quadruplex-interactive telomerase inhibitor, telomestatin (SOT-095), against human leukemia cells: involvement of ATM-dependent DNA damage response pathways. *Oncogene* 22 (34), 5338-47.

Tsui, N. B., Ng, E. K. and Lo, Y. M. (2002) Stability of endogenous and added RNA in blood specimens, serum, and plasma. *Clin Chem* 48 (10), 1647-53.

Uehara, H., Nardone, G., Nazarenko, I. and Hohman, R. J. (1999) Detection of telomerase activity utilizing energy transfer primers: comparison with gel- and ELISA-based detection. *Biotechniques* 26 (3), 552-8.

Uziel, O., Fenig, E., Nordenberg, J., Beery, E., Reshef, H., Sandbank, J., Birenbaum, M., Bakhanashvili, M., Yerushalmi, R., Luria, D. and Lahav, M. (2005) Imatinib mesylate (Gleevec) downregulates telomerase activity and inhibits proliferation in telomerase-expressing cell lines. *Br J Cancer* 92 (10), 1881-91.

Van Cutsem, E., Lenz, H. J., Köhne, C. H., Heinemann, V., Tejpar, S., Melezínek, I., Beier, F., Stroh, C., Rougier, P., van Krieken, J. H. and Ciardiello, F. (2015) Fluorouracil, leucovorin, and irinotecan plus cetuximab treatment and RAS mutations in colorectal cancer. *J Clin Oncol* 33 (7), 692-700.

van Delft, M. F., Wei, A. H., Mason, K. D., Vandenberg, C. J., Chen, L., Czabotar, P.

E., Willis, S. N., Scott, C. L., Day, C. L., Cory, S., Adams, J. M., Roberts, A. W. and Huang, D. C. (2006) The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* 10 (5), 389-99.

van der Riet, P., Nawroz, H., Hruban, R. H., Corio, R., Tokino, K., Koch, W. and Sidransky, D. (1994) Frequent loss of chromosome 9p21-22 early in head and neck cancer progression. *Cancer Res* 54 (5), 1156-8.

van der Schroeff, M. P. and Baatenburg de Jong, R. J. (2009) Staging and prognosis in head and neck cancer. *Oral Oncol* 45 (4-5), 356-60.

Vaziri, H. and Benchimol, S. (1998) Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr Biol* 8 (5), 279-82.

Vermorken, J. B., Mesia, R., Rivera, F., Remenar, E., Kawecki, A., Rottey, S., Erfan, J., Zabolotnyy, D., Kienzer, H. R., Cupissol, D., Peyrade, F., Benasso, M., Vynnychenko, I., De Raucourt, D., Bokemeyer, C., Schueler, A., Amellal, N. and Hitt, R. (2008) Platinum-based chemotherapy plus cetuximab in head and neck cancer. *N Engl J Med* 359 (11), 1116-27.

Villa, R., Folini, M., Porta, C. D., Valentini, A., Pennati, M., Daidone, M. G. and Zaffaroni, N. (2003) Inhibition of telomerase activity by geldanamycin and 17-allylamino, 17-demethoxygeldanamycin in human melanoma cells. *Carcinogenesis* 24 (5), 851-9.

Wang, Z., Song, W., Aboukameel, A., Mohammad, M., Wang, G., Banerjee, S., Kong, D., Wang, S., Sarkar, F. H. and Mohammad, R. M. (2008) TW-37, a small-molecule inhibitor of Bcl-2, inhibits cell growth and invasion in pancreatic cancer. *Int J Cancer* 123 (4), 958-66.

Warnakulasuriya, S. (2009) Global epidemiology of oral and oropharyngeal cancer. *Oral Oncol* 45 (4-5), 309-16.

Wheelhouse, R. T., Jennings, S. A., Phillips, V. A., Pletsas, D., Murphy, P. M., Garbett, N. C., Chaires, J. B. and Jenkins, T. C. (2006) Design, synthesis, and evaluation of novel biarylpyrimidines: a new class of ligand for unusual nucleic acid structures. *J Med Chem* 49 (17), 5187-98.

WHO (2014) *WHO Cancer. http://www.who.int/mediacentre/factsheets/fs297/en/* Accessed 31 May 2016.

WHO (2018) *Cancer statistics fact sheet.* World Health Organisation.

Xi, L. and Cech, T. R. (2014) Inventory of telomerase components in human cells reveals multiple subpopulations of hTR and hTERT. *Nucleic Acids Res* 42 (13), 8565-77.

Yamamoto, Y. and Hirakawa, E. (2005) Telomerase activity and hTERT mRNA expression in body fluids. *Diagn Cytopathol* 32 (3), 167-70.

Yu, C. C., Lo, S. C. and Wang, T. C. (2001) Telomerase is regulated by protein kinase C-zeta in human nasopharyngeal cancer cells. *Biochem J* 355 (Pt 2), 459-64.

Zahler, A. M., Williamson, J. R., Cech, T. R. and Prescott, D. M. (1991) Inhibition of telomerase by G-quartet DNA structures. *Nature* 350 (6320), 718-20.

Zhang, H., Dziegielewski, P. T., Biron, V. L., Szudek, J., Al-Qahatani, K. H., O'Connell, D. A., Harris, J. R. and Seikaly, H. (2013) Survival outcomes of patients with advanced oral cavity squamous cell carcinoma treated with multimodal therapy: a multi-institutional analysis. *J Otolaryngol Head Neck Surg* 42, 30.

Zhang, L., Ming, L. and Yu, J. (2007) BH3 mimetics to improve cancer therapy;

mechanisms and examples. *Drug Resist Updat* 10 (6), 207-17.

Zhang, R. G., Guo, L. X., Wang, X. W. and Xie, H. (2002) Telomerase inhibition and telomere loss in BEL-7404 human hepatoma cells treated with doxorubicin. *World J Gastroenterol* 8 (5), 827-31.

Zhang, T. C., Cao, E. H., Li, J. F., Ma, W. and Qin, J. F. (1999) Induction of apoptosis and inhibition of human gastric cancer MGC-803 cell growth by arsenic trioxide. *Eur J Cancer* 35 (8), 1258-63.

Zlotta, A. R., Fleshner, N. E. and Jewett, M. A. (2009) The management of BCG failure in non-muscle-invasive bladder cancer: an update. *Canadian Urological Association journal* 3 (6 Suppl 4), S199-S205.

6 APPENDIX

Appendix I

Ethics approval letter

Bradford Research Ethics Committee

NHS

Top Floor
Extension Block
St Lukes Hospital
Little Horton Lane
Bradford
BD5 0NA

Chairman: **Professor Alan C Roberts**
OBE TD DL MPhil PhD LLD,FLS CBiol FIBiol
Administrator: **Sue Bell**

Tel: 01274 365508
Fax: 01274 365509
Email: sue.bell@bradfordhospitals.nhs.uk
Email: alan.roberts@bradfordhositals.nhs.uk

24 March 2005

Mr David Watt
Consultant Plastic Surgeon
University of Bradford
Plastic Surgery and Burns Research Unit
Bradford
BD7 1DP

Dear Mr Watt

Full title of study: ***Is Telomerase Detected in Saliva a Useful Marker of Upper Aero-Digestive Tract Squamous Cell Cancer?***
REC reference number: ***05/Q1202/14***

Thank you for your letter of 10 March 2005, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chairman.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

However, the Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the research site(s) taking part in this study. The favourable opinion does not therefore apply to any site at present. I will write to you again as soon as one Local Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at sites requiring SSA.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type:	Version:	Dated:	Date Received:
Application		31/01/2005	31/01/2005
Protocol	1	01/02/2005	31/01/2005
Covering Letter		31/01/2005	31/01/2005
Copy of Questionnaire	1	01/02/2005	31/01/2005
Participant Information Sheet	2 Healthy Volunteers	10/03/2005	16/03/2005
Participant Information Sheet	2 Group 1	10/03/2005	16/03/2005
Participant Consent Form	2 Healthy Volunteers	10/03/2005	16/03/2005
Participant Consent Form	2	10/03/2005	16/03/2005
Participant Consent Form	2 Spare Tissue Consent	10/03/2005	16/03/2005
Response to Request for Further Information		10/03/2005	16/03/2005

Management approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final management approval from the R&D Department for the relevant NHS care organisation.

Notification of other bodies

The Committee Administrator will notify the research sponsor that the study has a favourable ethical opinion.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q1202/14	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

Professor A C Roberts
Chairman

Bradford Research Ethics Committee

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	05/Q1202/14	**Issue number:**	1	**Date of issue:**	24 March 2005
Chief Investigator:	Mr David Watt				
Full title of study:	Is Telomerase Detected in Saliva a Useful Marker of Upper Aero-Digestive Tract Squamous Cell Cancer?				

This study was given a favourable ethical opinion by Bradford Research Ethics Committee on 24 March 2005. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

Principal Investigator	Post	Research site	Site assessor	Date of favourable opinion for this site	Notes [1]
Mr David Watt		Mr David Watt University of Bradford Plastic Surgery and Burns Research Unit Bradford BD7 1DP	Bradford Research Ethics Committee	24/03/2005	

Approved by the Chair on behalf of the REC:

..... (Signature of Chair/Administrator*)
(**delete as applicable)*

Sue Bell
Administrator, Research Ethics Committee

..... (Name)

Appendix II

Information leaflet

UNIVERSITY OF BRADFORD

MAKING KNOWLEDGE WORK

Department of Biomedical Sciences
Plastic Surgery & Burns Unit

Head of Department
Dr S M Parkin BSc PhD

Director of Unit
Professor D T Sharpe OBE DT MA MB MChir FRCS

Research Fellows
Mr R O S Karoo MRCS
Mr W L Lam MRCS
Mr J M Rawlins MRCS

Study into using saliva for detection of Telomerase enzyme

We would very much appreciate your help in a research project

Before you decide, please take time to read the following information carefully and discuss it with friends, relatives or with your doctor at the Head and Neck outpatient clinic, Bradford Royal Infirmary.

What is the purpose of the study

The aim of the study is to test the presence of a certain enzyme in the saliva of patients found to have upper aero-digestive tract cancer. This enzyme is not normally found in saliva in healthy individuals and its presence can be used as guide in detecting upper aero-digestive tract cancer in people who are suspected of having the condition earlier.

How can you help?

You can help us by consenting to the following:-

- We will ask you for a sample of your saliva at your clinic visit so that it can be used for tests in the laboratory.
- During your operation, the surgeon will remove some tissue from your mouth or throat. A small amount of this tissue is examined in detail in the pathology department so that the pathologist can help with the diagnosis. Most of the tissue is however disposed of and it is a small part of this that we plan to use. No extra procedures are therefore involved in collecting the sample that we require for research.
- We plan to store these tissue samples for up to three years in the cancer research unit, Bradford where we will be studying a variety of enzymes present in them. Part of the research will also involve having access to the tissue stored in the pathology department after the pathologist has examined it.
- Finally we will also require information from you about your general health so that we can record accurate clinical details to go with the results of the research. Full confidentiality will be maintained in dealing with your tissue specimens and also in dealing with your clinical information. People qualified to do the job will handle them.
  We shall treat each sample collected as a gift towards the progress of research

INVESTOR IN PEOPLE

Richmond Road Bradford West Yorkshire BD7 1DP UK
Tel Direct Line 01274 233576/235524 General Office 01274 233560/233561 Fax 01274 309742
International Tel Direct Line +44 1274 233576/235524 General Office +44 1274 233560/233561 Fax +44 1274 309742
www.bradford.ac.uk

Do you have to take part?
No. It is up to you whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without a reason.

May we assure you that if you choose not to participate in this study, your future care at the BRI will not be affected in any way.

Will the research influence your current treatment?

If you take part in this study, it will not influence your current treatment.
Neither will you receive the results of research done with your tissue. This is because the project can take many years to complete and must use tissue samples from many people before results are known. Instead, your participation in this very important research study may well have an impact on how similar patients are treated in future.

What about confidentiality

Information about you will remain strictly confidential.

Your name, and any other identifying information will be taken out of anything associated with your tissue sample before it is given to the researcher.

A code number and not your name will identify your sample, and neither you nor your relatives will therefore be identified or contacted.

Also people outside the research process will not have the results about any one person. No individual person will be identified when the results of the research are published.

Finally what happens if you also give consent for any of the left over tissue being used in possible future research projects?

Sometimes not all of the collected tissue sample is used up during the research project and is therefore disposed of, even though it might be useful for new experiments that cannot presently be foreseen. This can be avoided however by consenting for its use in possible future research projects. There are many benefits of this not the least the more efficient use of these sample collections. Although the exact details of future studies are unknown it should be stressed that their goals would be similar to those being undertaken in this study.

Will similar safeguards be in place to protect your confidentiality?

Although a different group of researchers within the Plastic surgery and burns research unit and the Cancer research unit may be involved with any future work, the same safeguards would be in place to protect your rights.

What if I have any more questions?
If you have any more questions, please don't hesitate to contact your Consultant.

Thank you for taking time to read this information and to consider being involved in this research.

Appendix III

Consent form

UNIVERSITY OF BRADFORD

MAKING KNOWLEDGE WORK

Department of Biomedical Sciences
Plastic Surgery & Burns Unit

Head of Department
Dr S M Parkin BSc PhD

Director of Unit
Professor D T Sharpe OBE DT MA MB MChir FRCS

Research Fellows
Mr R O S Karoo MRCS
Mr W L Lam MRCS
Mr J M Rawlins MRCS

Consent form

Study using saliva for detection of Telomerase enzyme

1. I confirm that I have read and understood the information sheet entitled 'study using saliva for detection of telomerase enzyme'.

2. I understand that my participation is voluntary.

3. I consent to a saliva and tissue sample being collected.

4. I consent to allow the saliva and tissue sample to be used and stored up to 3 years for this research.

5. I am willing for information gathered in the questionnaire to be used providing that strict confidentiality is maintained.

Name of volunteer	**Date**	**Signature**
Name of person obtaining consent	**Date**	**Signature**

INVESTOR IN PEOPLE

Richmond Road Bradford West Yorkshire BD7 1DP UK
Tel Direct Line 01274 233576/235524 General Office 01274 233560/233561 Fax 01274 309742
International Tel Direct Line +44 1274 233576/235524 General Office +44 1274 233560/233561 Fax +44 1274 309742
www.bradford.ac.uk

Appendix IV

Questionnaire

Study using saliva to detect Telomerase

1. Name:

2. Age: Years

3. Sex: Male
Female

4. Do you smoke? No
Yes

If No, have you ever smoked No
Yes

Years smoker
Years/ weeks abstinence
Number per day

5. Do you drink alcohol? No
Yes

Units per week

6. Do you use dentures? No
Yes

7. Do you suffer from gum disease? No
Yes

8. Have you got a cold, catarrh or sore throat? No
Yes

9. Do you have any known medical conditions?

No
Yes

Please give details

10. Are you taking any medication?

No
Yes

Please give details

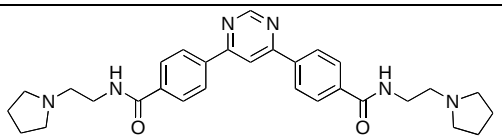
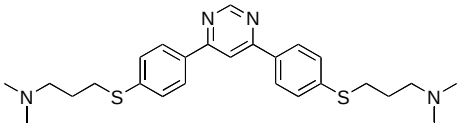
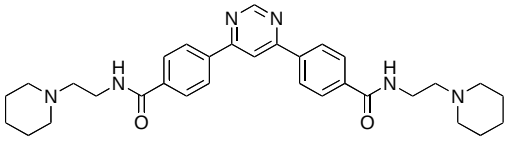
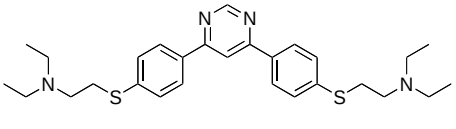
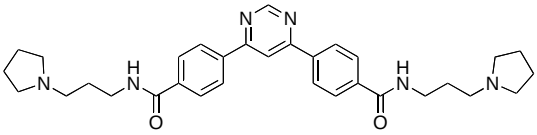
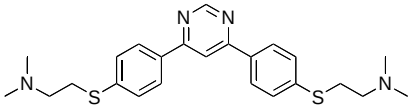
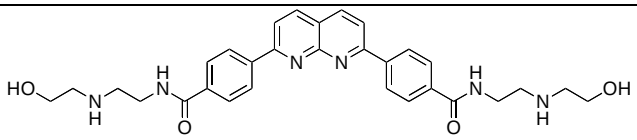
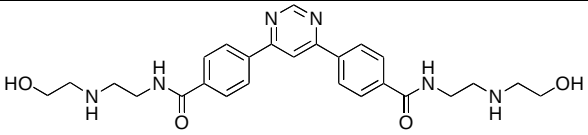
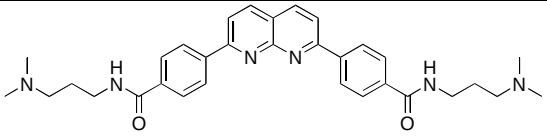
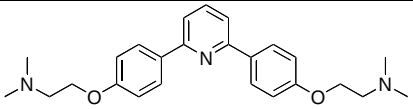
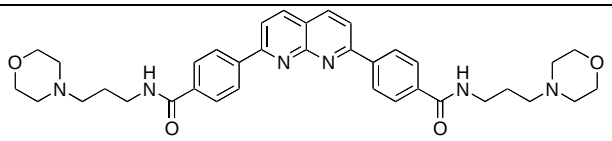
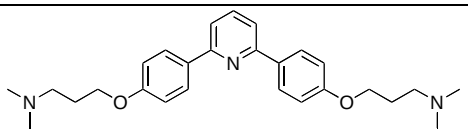
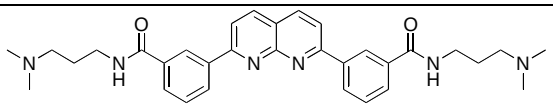
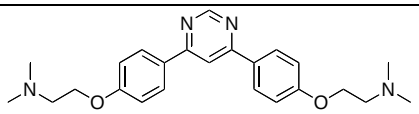
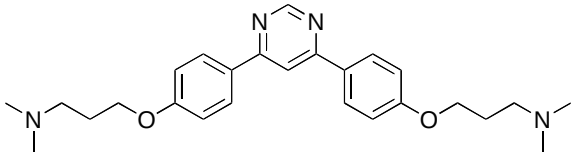
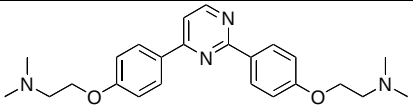
11. Are you currently involved in existing research, or have recently been involved?

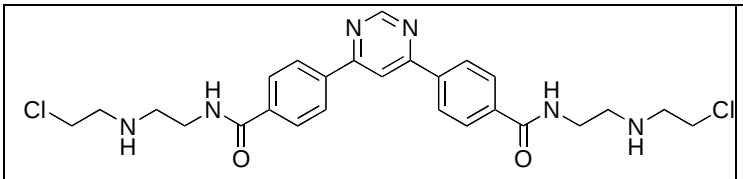
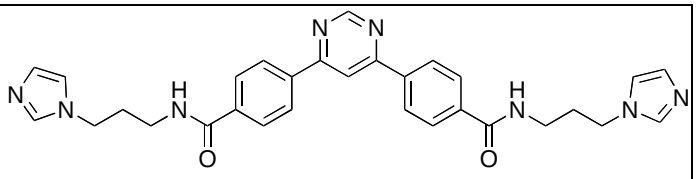
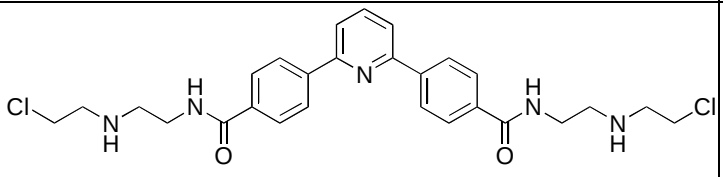
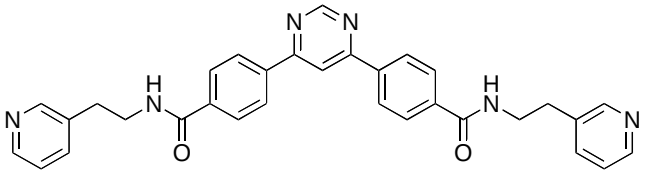
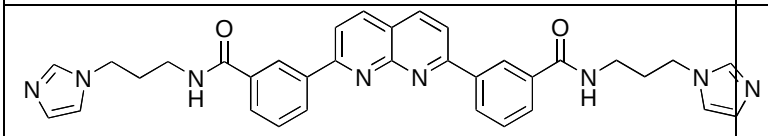
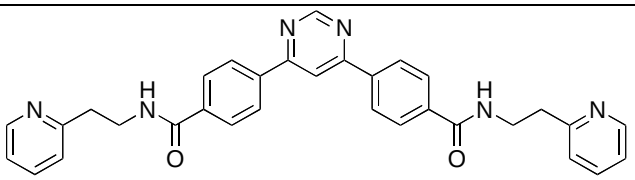
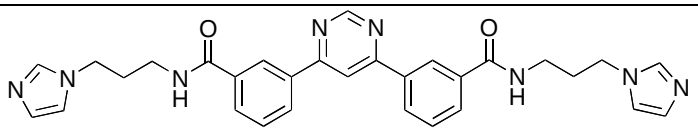
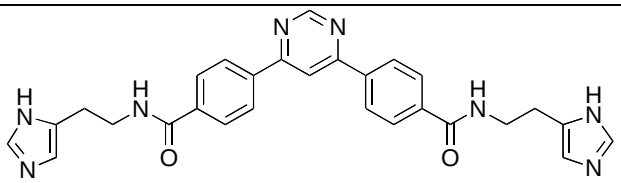
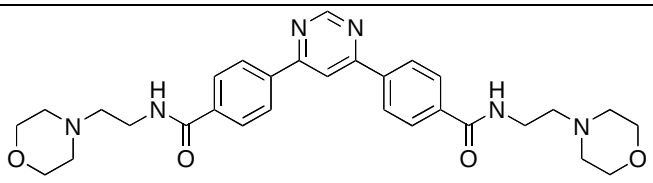
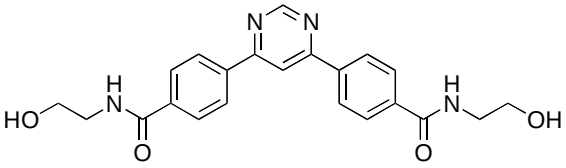
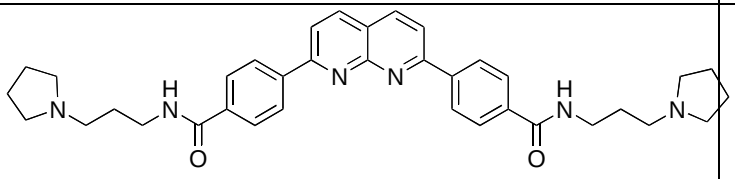
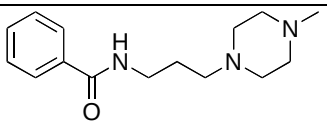
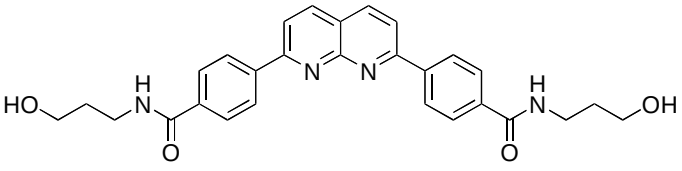
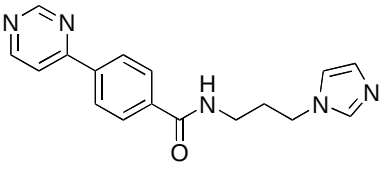
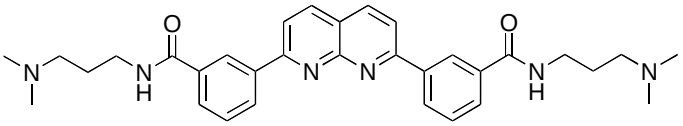
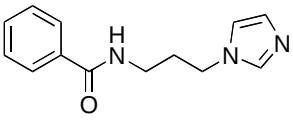
No
Yes

Thank you for your co-operation in filling this form

Appendix V

Compounds used in study

 VP1PRR2	 SJ45
 VP1PIP2	 LSCEt
 VP1PRR3	 LSCMe
 VP2MITOH	 VP1MITOH
 VP2ME3	 HE19
 VP2MPH	 HE20
 mVP2ME3	 HE24
 HE25	 HE27

 VP1MITCl	 RW0071A
 VP3MITCl	 RW0073
 mVP2IMI3	 RW0071B
 mVP1IMI3	 RW0071D
 VP1MPH2	 RW0041
 VP2PRR3	 RW0020
 VP2OH3	 RW0029
 mVP2Me3	 RW0016

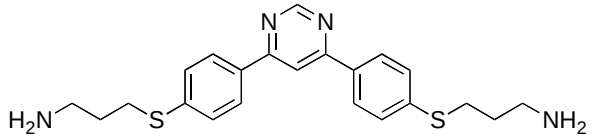
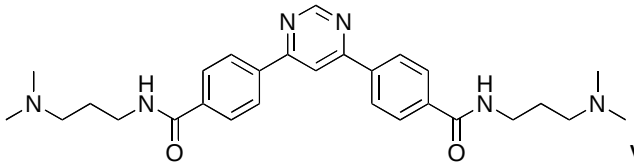
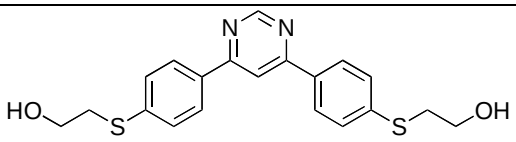
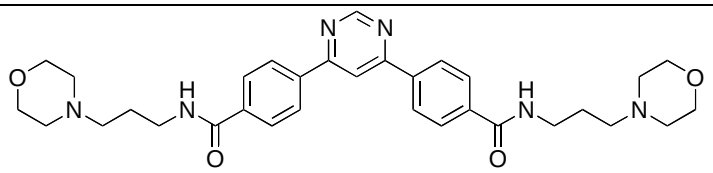
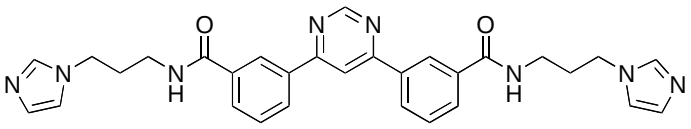
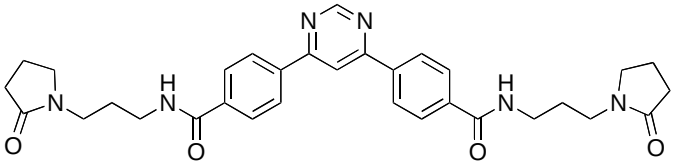
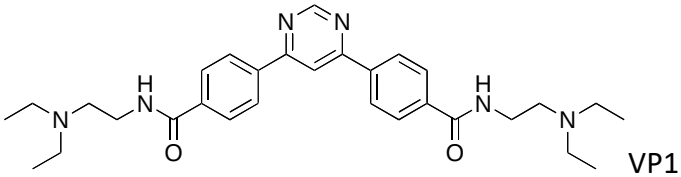
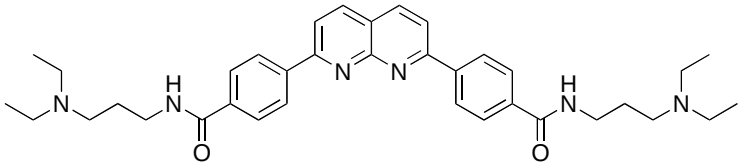
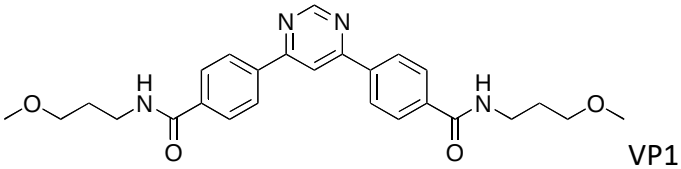
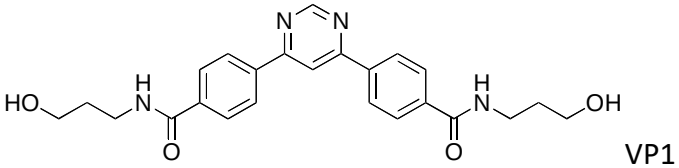
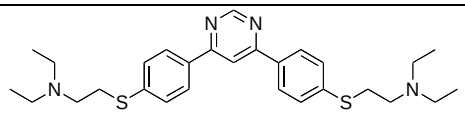
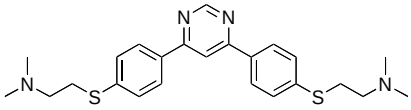
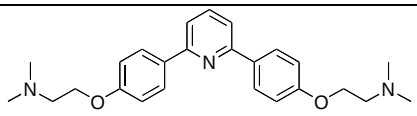
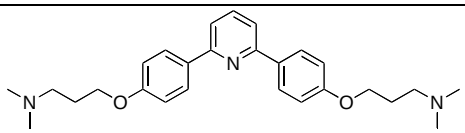
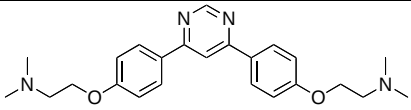
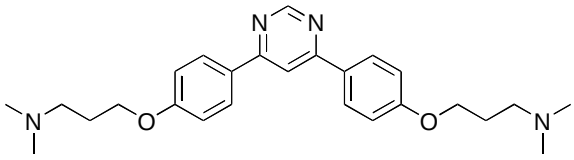
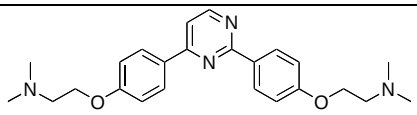
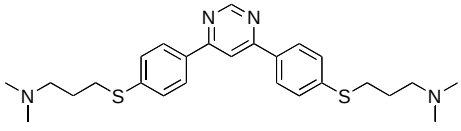
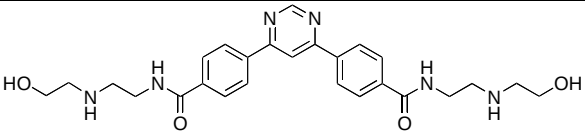
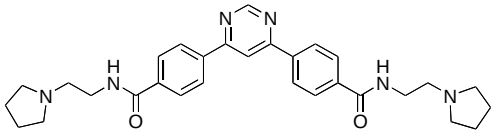
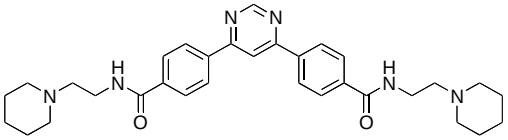
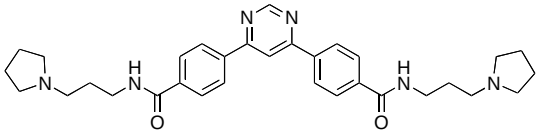
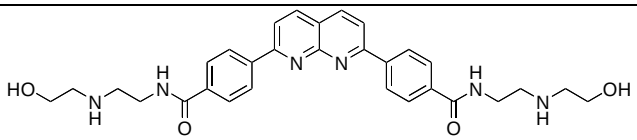
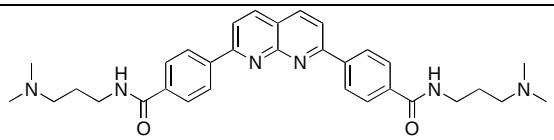
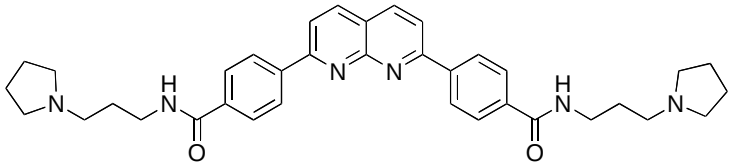
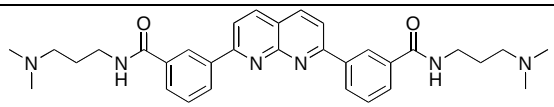
 SJ44	 VP1ME3
 LSCOH	 VP1MPH3
 mVP1IMI3	 VP1LCT
 VP1 Et2	 VP2Et3
 VP1 OMe3	
 VP1 OH3	

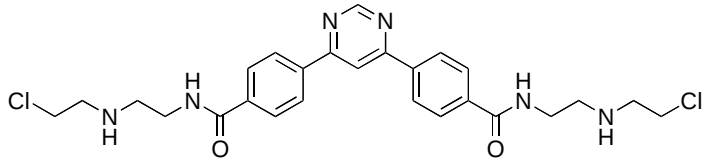
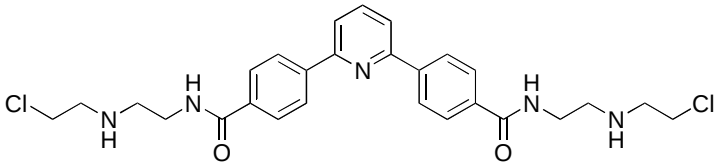
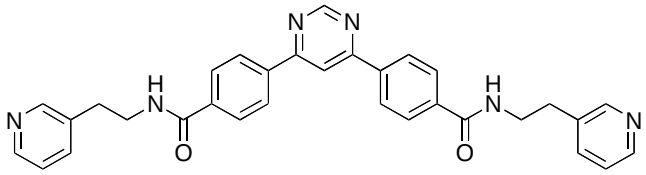
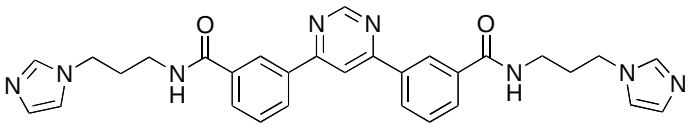
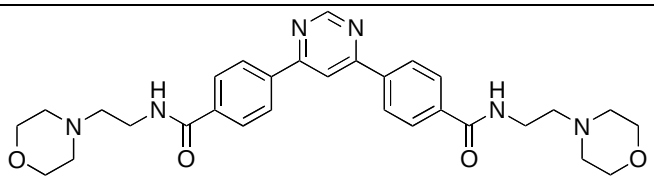
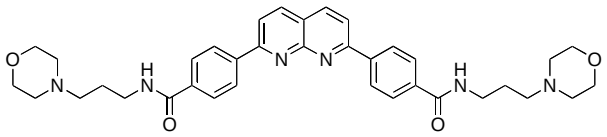
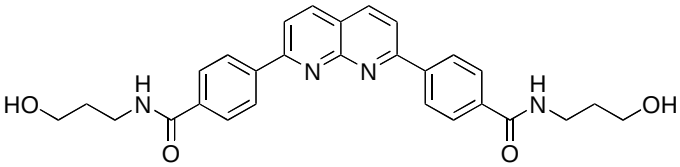
Table 14 Structures of compounds used in study

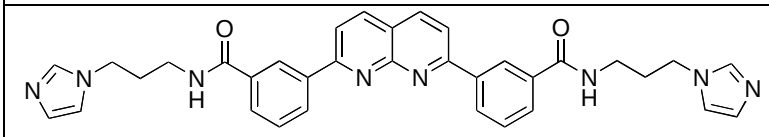
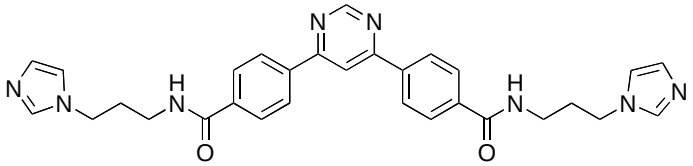
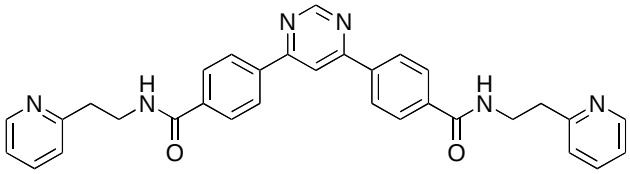
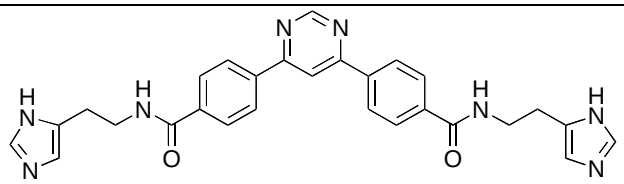
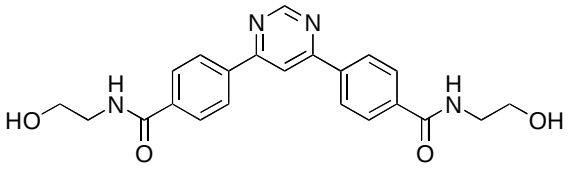
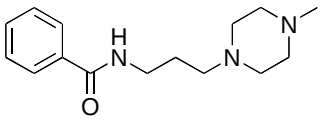
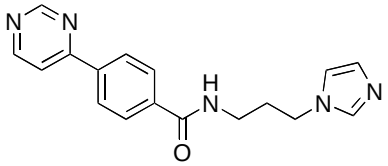
Appendix VI

Compounds used in study and their effect on telomerase and Taq polymerase

COMPOUND	Telomerase inhibitor	Taq inhibitor
 LSCEt	Y (IC50 – 89)	N
 LSCMe	Y (IC50 – 102)	N
 HE19	Y (IC50 – 140)	N
 HE20	Y (IC50 – 165)	N
 HE24	Y	Y
 HE25	Y	Y
 HE27	Y	Y
 SJ45	Y	Y
 VP1MITOH	Y	Y

COMPOUND	Telomerase inhibitor	Taq inhibitor
 VP1PRR2	Y	Y
 VP1PIP2	Y	Y
 VP1PRR3	Y	Y
 VP2MITOH	Y	Y
 VP2ME3	Y	Y
 VP2PRR3	Y	Y
 mVP2ME3	Y	Y

COMPOUND	Telomerase inhibitor	Taq inhibitor
 VP1MITCl	Y	Y
 VP3MITCl	Y	Y
 RW0073	Y	Y
 mVP1IMI3	N	-
 VP1MPH2	N	-
 VP2MPH	N	-
 VP2OH3	N	-

COMPOUND	Telomerase inhibitor	Taq inhibitor
 mVP2IMI3	N	-
 RW0071A	N	-
 RW0071B	N	-
 RW0071D	N	-
 RW0041	N	-
 RW0020	N	-
 RW0029	N	-

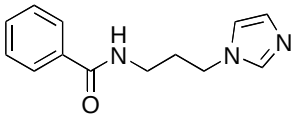
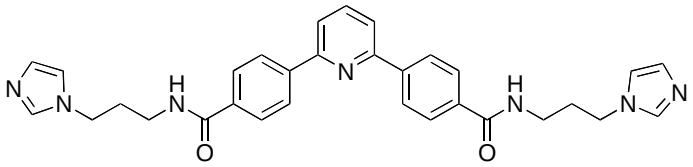
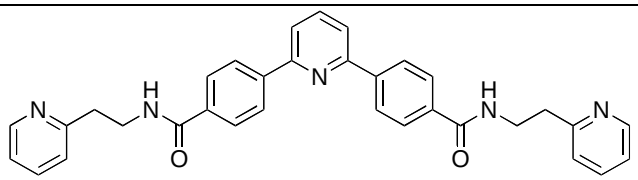
COMPOUND	Telomerase inhibitor	Taq inhibitor
 RW0016	N	-
 AC-A	N	-
 AC-B	N	-

Table 15 Structures of 34 compounds used in study and their effect on telomerase and Taq polymerase